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Dated 27 April 2004

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P01/7700 0.00-0307559.5

Patents Form 1/77

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Request for grant of a patent

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1. Your reference ~~P3132 GB PRO~~

2. Patent application number *(The Patent Office will fill in this part)* 02 APR 2003 0307559.5

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

UNIVERSITY OF NOTTINGHAM
UNIVERSITY PARK
NOTTINGHAM
NG7 2RD

Patents ADP number *(if you know it)* 00798405001

If the applicant is a corporate body, give the country/state of its incorporation UNITED KINGDOM (ENGLAND)

4. Title of the invention FLUORESCENTLY TAGGED LIGANDS

5. Name of your agent *(if you have one)* NOVAGRAAF PATENTS LIMITED

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

THE CRESCENT
54 BLOSSOM STREET
YORK YO24 1AP

Patents ADP number *(if you know it)* 08299166001 ✓

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	77
Claim(s)	~~26~~ 27
Abstract	1
Drawing(s)	1 + See minute

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Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	ONE
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Novagraaf Patents Ltd Date

NOVAGRAAF PATENTS LIMITED 01/04/2003

12. Name and daytime telephone number of person to contact in the United Kingdom

ALLEN, PHILIPPA M (GB)

01904 610586

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FLUORESCENTLY TAGGED LIGANDS

The present invention relates to a kit comprising a plurality of tagged non-peptide ligands in which a precursor ligand is linked to a number of different tags or a plurality of precursor ligands are each linked to one or a plurality of different tags; known and novel tagged ligands associated with information on their pharmacology; novel tagged ligands; reactive non-peptide ligands and at least one reactive tagging substrate suitable for the preparation of known and novel tagged ligands and kits thereof; processes for the preparation of known and novel tagged ligands and kits of ligands; the use of known and novel tagged ligands and kits of tagged ligands in studying receptor binding such as biogenic amine G-protein coupled receptor (GPCR) binding or intracellular enzyme inhibition such as cyclic nucleotide phosphodiesterase inhibition. More specifically the invention relates to a kit comprising a family of tagged non-peptide ligands in which a precursor ligand is linked to a number of different tags one or all of which is a fluorescent tag or a plurality of precursor ligands are each linked to one fluorescent tag or to a plurality of different fluorescent tags; known and novel fluorescently tagged ligands associated with information on their pharmacology; novel fluorescently tagged ligands; reactive non-peptide ligands and at least one reactive fluorescent tagging substrate suitable for the preparation of known and novel fluorescently tagged ligands and kits thereof; processes for the preparation of known and novel fluorescently tagged ligands and kits of fluorescently tagged ligands in high yield; and the use of known and novel fluorescently tagged ligands and kits of fluorescently tagged ligands in studying receptor binding such as biogenic amine G-protein coupled receptor (GPCR) binding or intracellular enzyme inhibition such as cyclic nucleotide phosphodiesterase inhibition in cell populations or single cells such as acutely dispersed cells using techniques

such as Confocal Microscopy and Fluorescence Activated Sorting and Fluorescence Correlation Microscopy.

Jacobsen *et al* J Med Chem, 1985, 28, 1334 – 1340 disclose a functionalised congener approach to preparing 1,3-dialkylxanthine antagonists having increased potency, prolonged duration of action and/or changed specificity, and investigates binding affinity to A_1 adenosine and beta-adrenergic receptors. Derivatives of adenosine receptor agonists (*N6*-phenyladenosines) and antagonists (1,3-dialkyl-8-phenylxanthines) are disclosed with functionalised side chains suitable for radiolabelling or biotinylating with a view to preparing receptor probes. XAC (xanthine amine congener) was highlighted as the most potent agonist with high water solubility and its suitability as a key intermediate for preparation of i.a fluorescent probes was postulated.

Jacobsen *et al*, Biochemical Pharmacology, Vol 36, No 10, 1697 – 1707, 1987 extends the functionalised congener approach of earlier work to synthesising non-radioactive molecular probes for drug binding sites. Probes were synthesised from an antagonist XAC and an agonist adenosine amine congener (ADAC). Fluorescent dyes used for synthesising the probe included fluorescein, 4-nitrobenz-2-oxa-1,3-diazole (NBD) and Rhodamine. Competitive binding assays showed binding affinity of the probes. Fluorescence detection is not reported but potential applications in fluorescent cell sorting (FACS) to identify cell populations with a high density of receptors and antibody quenching to investigate accessability is postulated.

McGrath *et al TiPS* November 1996 (Vol 17) 393 – 399 reviews the possibilities for using fluorescent ligands in place of more traditional radioactive ligands in the study of cell receptors to report the amount of

ligand-receptor complex indicating the number of receptors, using traditional techniques of confocal spectroscopy and fluorescence activated cell sorting (FACS). He states that many attempts have been made at conjugating fluorescent molecules to receptor ligands in the hope of identifying their binding sites, aimed mainly at localisation of the receptors rather than studying their properties. Some compounds were available that fluoresce when bound to a receptor but which gave low background noise in the aqueous phase. The objective was to produce a fluorescent drug which would remain fluorescent when bound to the receptor and would remain bound when unbound drug was washed away. Therefore there was a need for very high receptor binding affinity. There was also a need to assess pharmacological properties of fluorescent drugs to check that they are similar to the parent drug. Reviewed work includes fluorescent ligand binding to nicotinic receptors, beta adrenoceptors (fluorescent acridinyl and dansyl analogues of propanolol (9-AAP, 9-amino acridino propanolol) and DAPN, DL-N-(2-hydroxy-3-naphthlyoxy propyl)N'-dansyl ethylenediamine) of which fluorescence was thought to be non-specific to receptors but showing non-fluorescent granules, and using BODIPY-CGP12177 where fluorescence was observed: no pharmacology was investigated; opioid GPCR type receptors (eg fluorescent labelled enkephalin), histamine (eg fluorescein-histamine binding), neurotensin (eg N-alpha-FTC-[Glu']NT (fluo-NT) and alpha-adrenoceptors (using a BODIPY-quinazoline, QAP-B or BODIPY FL-prazosin). The publication also reviews benefits of confocal microscopy. Efforts made to study the pharmacological properties of the ligands are reported in the case of only a few of the above notably comparing affinity of BODIPY-CGP12177 against FITC (fluorescein isothiocyanate)-CGP12177 and a comment is made that the choice of substrate drug therefore becomes an important factor in the synthesis of any putative fluoro-ligand.

However very few efforts to visualise receptors or classes of receptors have been shown to work. McGrath also mentions the possibility to visualise intracellular binding sites using a ligand which penetrates the cell and suggests that QAP-B can be shown to enter intact cells, whilst BODIPY CGP12177 remains a choice for surface beta-adrenoceptors due to its hydrophilic nature.

Pharmacological properties are usually to some extent affected by linking of a fluorophore to any receptor binding ligand, and include change in binding affinity, and in activation or otherwise of receptor, ie agonist or antagonist properties. It is important that the pharmacology of the fluorescent ligand is known in any studies in order to quantify the binding results observed.

The adenosine-A_1 receptor (A_1-AR) is a GPCR which is found in a variety of tissues including brain, heart, adipose tissue and muscle, and has been implicated in the pathophysiology of a number of conditions (Ralevic, V. and Burnstock, J (1998) Pharmacol. Rev. 50, 415).

Currently the study of A_1-AR pharmacology can only be performed well in cells which can be grown in large numbers using for example techniques such as radioligand binding. Autoradiography enables single cell studies but does not allow direct reading of binding and can take up to 4 - 6 weeks to develop the film to obtain results of binding. To overcome this problem, a very few fluorescent ligands of the invention have been adapted for use in visualising receptors and obtaining quantitative receptor-ligand binding data in single cells, using confocal microscopy (CM) and fluorescence correlation spectroscopy (FCS).

Confocal microscopy allows visualisation of a section through a cell showing concentration of fluorophore at the cell edges indicating membrane receptor

binding. Different coloured channels may be selected to visualise different fluorophore types. FCS analyses the diffusion characteristics of fluorescent species through a very small excitation volume ($<10^{-15}$l) by statistically analysing the pattern of their photon emissions. Thus fast-diffusing free ligand can be distinguished from slowly-diffusing receptor-bound ligand and quantified simultaneously when the volume is localised to the cell membrane.

Auer *et al* (Drug Discovery Today, Vol 3, No 10, Oct 98, 457- 465) present Fluorescence Correlation Spectroscopy (FCS) as a more or less unique solution to miniaturised assays, enabling *in vitro* high throughput screening (HTS) and ultimately enabling hit profiling in the native cellular context). In one of a number of examples of FCS binding assays GPCR's are presented as possibly the single most important class of targets for current and prospective drug therapies and reference is made to only a few successful examples of minituarised assays for ligand binding to these receptors. Auer *et al* disclose FCS ligand binding assays constructed for GPCR receptors giving as example binding to CD45 phosphatase receptor using a modified ELISA binding substrate, TMR fluorescence labelled phosphotyrosine peptide, and inhibitor binding monitored by FCS.

Macchia *et al* in Bioinorganic and Medicinal Chemistry Letters 8 (1998) 3223-3228 investigates fluorescence microscopy for its ability to localise receptors at single cell or sub cellular level, high sensitivity and speed whereby they postulate that high affinity molecular probes could help to elucidate molecular characteristics of adenosine receptor subtypes, their regional distribution and cellular localisation. For that purpose they developed NECA-dansyl probes having alkyl spacers of C_3-C_{12}. However they conducted only radioligand binding assays. More recently Macchia *et al* in Bioinorganic and Medicinal Chemistry Letters 11 (2001) 3023-3026 investigates receptor

ligand complexes without resorting to earlier practices of fixing cells to detect receptors, by using confocal microscopy, and discloses new fluorescent ligands for adenosine receptors obtained by insertion in the N_6 position of NECA, a potent non-selective adenosine agonist, of NBD fluorophores with linear alkyl diamine spacers of increasing length from C_3 to C_{12}. The ligands show a high affinity and selectivity for the A_3 subtype expressed in CHO cells. Again they conducted radiobinding assays and, in this case, also fluorescence microscopy assays which permitted visualisation and localisation of the human receptor subtype using the most active and selective ligand (linker n = 8 or 6) but visualisation was poor. In Il Farmaco (2002), 57, 783-6 Macchia discloses 1,8-naphthpyridine-dansyl as A_1 selective, but with no function disclosed. Again the fluorescent ligands prepared by Macchia gave poor visualisation of binding probably due to the chosen fluorophores (dansyl and NBD) giving autofluorescence.

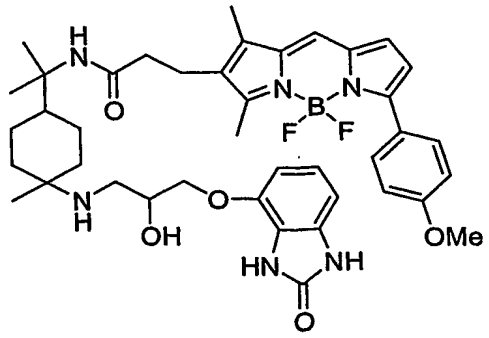
Fluorescent probes are known as above for attachment to only a few non peptide based receptor binding ligands. Molecular Probes have developed a very extensive class of dipyrromethane boron difluoride (BDI) probes for labelling in particular proteins and peptides. US 5,274,113 discloses BDI dyes for the fluorescent labelling of substances including cells, drugs etc to identify biological compounds in body fluids, cells and cell components. The dyes are unique in presenting a fluorescein like spectra, chemical reactivity with the functional groups typically combined with fluorescent tracers and solubility in non-polar environments. In US 5,274,113 and US 4,774,339 modified BDI dyes comprise the reactive functional group attached directly to the fluorophore or via a spacer – Lm- where m=0, 1 and L is C_{1-5} alkyl, and functional group is CO_2H and derivatives, alcohol or amine. US 6,323,186 (Molecular Probes) discloses the use of polyazaindacene dyes of BDI (dipyrromethaneboron difluoride) class, modified by linking to a phosphate

group of a nucleotide such as a purine or pyrimidine phosphate for the fluorescent labelling of nucleotides and assaying their binding to a protein or assaying for enzymatic cleavage of the nucleotide. Binding may be to a GPCR. In this case a linker moiety is a single covalent bond or includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine. When introduced into cells, the labelled nucleotide is generally introduced by pressure microinjection methods and other methods to physically permeabilise the plasma membrane.

Commercial fluorophores include BODIPY ™ 630/650 which comprises an internal alkyl linker and is known for use in tagging various peptides and proteins.

There are however few commercial non peptide fluorescent ligands for cell surface receptors that have been synthesised with BODIPY as the fluorophore.

The few commercial materials include histamine-BODIPY ™ FL and (pictured below) CGP12177-BODIPY ™ TMR (Molecular Probes):



However the synthesis of fluorescent ligands for the biogenic amine family of G-protein-coupled receptors presents serious problems. The BODIPY ™ fluorophores were initially designed for attaching to proteins which present a

much more uniform prospect for attaching: kits are available comprising a fluorophore and a set of reagents for universally attaching to most proteins. These give non specific attachments to any reactive site on the protein of interest and usually there is no need to know the nature or location of the attachment. However these proteins are larger molecules than the drugs (XAC etc) envisaged in the present invention. The ligand binding site for the biogenic amine GPCR receptors is also usually deep within the transmembrane regions of the receptor and thus the challenge is to attach to the fluorophore in such a way as to retain pharmacological activity. None of these BDI patents are concerned with the specific design of fluorescent agonists/antagonists with defined properties at GPCR's but rather with the fluorophore as an "add-on" probe.

In summary therefore the availability of fluorescent ligands and in particular non-peptide fluorescent ligands suitable for FCS and CM binding studies is virtually non-existant. The preparation of such compounds is far from routine and few efforts have been made to establish pharmacology. McGrath above only looked at a few of the receptor types studied.

There is moreover no unified approach in much of the prior art. Individual research has addressed fluorescent ligand systems which are limited to specific drug classes and or to the use of specific fluorophores. Such systems are limiting in both the information which can be obtained and in the number of systems which can be investigated.

Accordingly there is a need for novel fluorescent ligands giving reliable and effective receptor visualisation, of known pharmacological activity and receptor selectivity.

It is an object of the invention to provide novel fluorescent ligands for binding at desired receptors with established pharmacology in terms of both affinity and agonist and antagonist properties.

It is a further object of the invention to provide novel fluorescently tagged ligands that are linked to a number of different fluorophores (of different chemical composition, spectral characteristics etc) to provide a kit of known and novel fluorescent ligands with defined pharmacological characteristics.

It is a further object of the invention to provide an improved process for the design and preparation of known and novel fluorescent ligands, more specifically to provide non-peptide ligand precursors comprising chemical functionality for linking to any fluorophore to provide known or novel fluorescent ligands with linking at a desired site ensuring retention of receptor binding capability and linking in manner not to interfere with receptor binding capability, or to modify binding capability in known manner.

It is a further object of the invention to provide novel modified ligands including a linker moiety providing improved properties such as water solubility, on linking to a fluorescent moiety or any other desired non-hydrophilic probe.

It is a further object of the invention to provide improved fluorescent ligands for receptor binding having improved properties such as water solubility.

The innovation of the present invention relates to the design of specifically fluorescent "drugs" with known pharmacological properties. A key to this success is that each fluorophore has a specific influence on the pharmacology of the resulting product, and it is incorrect to assume that the compound will retain the properties of the non-fluorescent derivative. The invention therefore

provides defined and well characterized ligands, having verified properties corresponding to those of the non-fluorescent derivative.

In the broadest aspect of the invention there is provided a kit comprising a plurality of tagged non-peptide ligands of formula

Lig-L-Tag

in each of which a precursor ligand is linked to one of a plurality of different tags or a plurality of precursor ligands are each linked to a plurality of different tags

Wherein Lig- comprises a biogenic amine GPCR receptor ligand selected from a) an adenosine receptor antagonist b) an adenosine receptor agonist c) a beta-adrenoceptor agonist and d) a beta-adrenoceptor antagonist; or Lig- comprises e) an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases; or a derivative or analogue thereof;

-L- is a single bond or is any linking moiety which may be monomeric, oligomeric having oligomeric repeat of 2 to 30 or polymeric having polymeric repeat in excess of 30 up to 300;

and wherein -Tag is any known or novel tagging substrate.

-Tag may comprise any entity which is known in the art of tagging molecules to form a marker or reporter group for detecting molecules and which may be used in analytical studies relating to the ligand, and includes but is not limited to fluorophore tags as known in the art. In a particular advantage -Tag is a chemical entity which might be anticipated to inhibit receptor ligand binding or to inhibit intracellular enzyme inhibition in or by a compound Lig-L-Tag, and such inhibition is negated or dispelled by the presence of group –L-.

Preferably one or more or each -Tag is an entity -Fl and comprises any known or novel fluorophore, whereby the kit comprises a family or library of compounds of which one or more or all of which are of formula Lig-L-Fl.

Preferably the kit comprises one or more families of tagged non-peptide ligands in which one or more precursor ligand is linked to a number of different tags or comprises a library of non-peptide ligands in which a plurality of precursor ligands are each linked to one or a plurality of different tags as hereinbefore defined. Preferably a precursor ligand is linked to each of a plurality of fluorophores and/or tags providing a family of differently fluorescently tagged ligands; and/or providing a family of differently tagged ligands including at least one fluorescently tagged ligand. Alternatively a plurality of precursor ligands may be linked each to one or a plurality of different tags providing a library of same or differently tagged ligands of plural ligand type.

The kit may comprise from 2 to 250 tagged ligands. Preferably the kit comprises from 1 to 10 families comprising 2 to 25 tagged ligands each family comprising a ligand of a common ligand type and from 2 to 25 different tag types at least one of which is a fluorescent tag, more preferably each of which is a different fluorescent tag; or the kit comprises a library of from 5 to 250 fluorescently tagged ligands of different ligand type and different fluorophore type.

A kit providing fluorescent ligands linked to different Fl is useful to enable studying binding with different colour fluorescence for example to distinguish from same colour native fluorescence or to distinguish plural types of binding site or the like.

It is known that ligands modified ie by linking to a fluorophore are characterised by a change in binding affinity and suitably the kit of the invention comprises characterisation of the pharmacology of each Lig-L-Tag enabling quantification of results. Preferably the kit includes information for each tagged ligand Lig-L-Tag comprised in the kit, relating to the pharmacology for binding to a cell receptor of the biogenic amine class of GPCR receptors or to inhibition of intracellular enzymes such as cyclic nucleotide phosphodiesterases, including designation as agonist or antagonist and measure of affinity or inhibition etc.

In the prior art methods of preparing ligands the linking sites have in many cases been non-specific (Molecular Probes), or at best have been specific but possibly not rationalised for a desired effect. Preferably in the kit of the invention tagged ligands comprise fluorophores linked at any of a number of linking sites at which ligand receptor binding is maintained to a greater extent or is modified or inhibited to a lesser extent. Preferably the kit comprises tagged ligands designed from reaction of reactive precursor ligand(s) and reactive fluorophores having reactive site chemical functionality and suited for reaction with associated reagents, for site specific reaction and linking, wherein the design is the result of extensive investigation of all or many of the possible linking sites and the resulting pharmacological characteristics and selection of one or more linking combinations which provide favorable binding characteristics.

Preferably Lig- is selected from

a) xanthine like structures including XAC, theophyline, caffeine, theobromine, dyphilline, enprofylline and the like; or fused biaryl structures including papaverine, dihydroquinilones such as cilostamide, dipyridamole, vinpocetine and the like; and analogues thereof;

b) adenosine like structures including ADAC, ABEA, NECA and analogues thereof;

c) ethanolamine structures including Salmeterol, salbutamol, terbutaline, quinprenaline, labetalol, sotalol, bambuterol, fenoterol, reprotolol, tulobuterol, clenbuterol and analogues thereof;

d) oxypropanolamine structures including CGP12177, propranolol, practolol, acebutalol, betaxolol, ICI 118551, alprenolol, celiprolol (celectol), metoprolol (betaloc), CGP20712A, atenolol, bisoprolol, misaprolol, carvedilol, bucindolol, esmolol, nadolol, nebivolol, oxprenolol, xamoterol, pindolol, timolol and analogues thereof;

e) xanthine like structures including XAC, theophylline, caffeine, theobromine, dyphilline, enprofylline, sildenafil, EHNA (erythro-9-(2-hydroxyl-3-nonyl)adenine), zaprinast and the like; or spiro bicyclic structures including bypyridines such as amrinone, imidazolines such as CI930, dihydropyridazinones such as indolan, rolipram, SB207499, and the like; or fused biaryl structures including papaverine, dihydroquinilones such as cilostamide, dipyridamole, vinpocetine and the like and analogues thereof.

Linker –L- prevents loss of affinity of a ligand when modified to comprise a fluorescent moiety, as with the "spacers" known in the art and described by Macchia *et al* and Molecular Probes above, by distancing the fluorophore moiety from the Ligand binding structure, in cases that modifying by direct linking of Lig- and $-Fl_L$ would interfere with ligand binding, in which case a linker is usually a short, medium or long chain structure.

Linker Lig–L-Tag may optionally comprise functionality derived from its synthesis by the reaction of one reactive end group of a linker precursor with a reactive group of a ligand precursor and reaction of another reactive end group of the linker precursor with a reactive group of a tag precursor such as a

fluorescent tag precursor, or by the reaction of component linker moieties with a ligand precursor and interconversion as appropriate.

In a particular advantage of the present invention linker –L- facilitates linking of fluorescent moiety and ligand, in cases that groups of respective moieties are not reactive, or that stereochemistry or other effects inhibit linking, or that reaction of existing reactive groups in commercially available precursor ligands and fluorophores would require the inclusion of protecting groups for functionalities present therein, in which case a linker is usually a difunctional short, medium or long chain structure. In a further advantage of the invention linker –L- may confer properties facilitating crossing the cell membrane, hydrophobicity, hydrophilicity and the like as required, in which case a linker is usually any functionalised structure.

Preferably – L- is selected from a saturated or unsaturated single or double bond, -O-, -S-, amino, COO-, hydrazine; and saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, and comprising functionality derived from a reactive group or site for linking to fluorophore and/or to ligand selected from a saturated or unsaturated single or double bond, -O-, -S-, amino, hydrazine, carbonyl and the like. In the case that – L- comprises a single or double bond, then functionality derived from a reactive group or site for linking linker and fluorophore is usually present on the fluorescent moiety and/or on the ligand moiety.

More preferably –L- is selected from a single bond, -O-, -S-, amino; and C_{1-12} alkyl, alkenyl, alkynyl, alkoxy, amino, cycloalkyl, heterocyclic, aryl, heteroaryl, and combinations thereof such as aralkyl, wherein heteroatoms are as hereinbefore defined, optionally substituted as hereinbefore defined wherein substituents are selected from and C_{1-6} aliphatic, aromatic or alicyclic substituents as defined, hydroxy, thiol, halo, amine, oxo, and the like.

Suitably – L- is characterised by a short, medium or long chain moiety, and functionality derived from its synthesis by reacting a linker precursor and one or both of a ligand precursor and a fluorescent tag precursor comprises amino, -O-, -S-, alkoxy, thioxy, -COO-, oxo, amido and the like, whereby -L- may for example comprise an amino alkylthio, amino alkoxy, alkoxy carboxylic acid, alkoxy amine and the like. Preferably –L- is selected from mono or diamino menthane, mono or diamino ethane, mono or dithio ethane, mono or dihydroxy ethane, amino acid or polypeptide, or from mono or polyether diamine or dithio such as mono or polyethylene glycol diamine or dithio.

Preferably a linker –L- as hereinbefore defined retaining pharmacological activity comprises a mono or difunctional linear or cyclic substituted or unsubstituted alkyl of formula –L.I-

-L.I-

$$-Y_L'\left[(CR_L)q_L\ A\right]q_L''\ (CR_L)q_L'\ Y_L-$$

wherein each of $-Y_L'-$ and $-Y_L-$ is independently selected from a single bond, methylene, alkyne, alkene, NH, NR, O, NRCO, S, CO, NCO, CHHal, P and the like wherein Hal is any halogen selected from chlorine, iodine, fluorine;

-A- is a property conferring group selected from –O-, -C(=O)-cycloalkyl, heterocyclic, alkenyl, aryl, heteroaryl as hereinbefore defined and combinations thereof and the like;

q_L and q_L' are independently zero or are selected from a whole number integer from 1 to 10, preferably the sum of q_L and q_L' is zero, 1 or greater than or equal to 2 and less than or equal to 12; and

q_L'' is 0 or 1 to 30, or indicates a polymeric repeat unit of 30 up to 300

and each R_L is selected independently from H, C_{1-3} alkyl, C_{1-5} alkoxy and the like.

Preferably -L- is of formula -L.II-

-L.II- $-Y_L'[(CH_2)_2O]q_L''(CH_2)_2Y_L-$

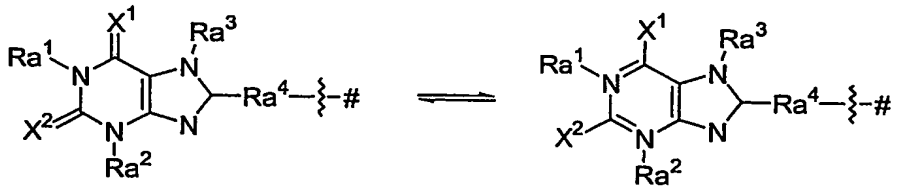
wherein Y_L', Y_L and q_L'' are as hereinbefore defined.

More preferably each Lig-L-Tag in a kit of the present invention comprises moiety Lig-L- selected from formulae Lig.a-L.a- – Lig.e- L.e-:

Wherein:

Lig.a- is suitably of the formula, in either of the following forms given:

Lig.a [1]-



Wherein X^1 and X^2 are each independently selected from H, =O, OR.a, NR.a, NHR.a;

X^1 and X^2 are each preferably =O;

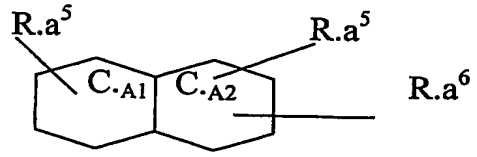
each of R.a, $R.a^1$, $R.a^2$ and $R.a^3$ independently is selected from H or C_{1-4} linear or branched alkyl, preferably H, methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl or isobutyl optionally mono or multi hydroxy or halo substituted, such as CH_2OH, CH_2F or $CH_2CHOHCH_2OH$;

$R.a^4$ is selected from a heteroatom O, S or substituted or unsubstituted amine or saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like;

preferably $R.a^4$ is selected from optionally substituted aryl, cycloalkyl, alkyl, ketone, (di)amine, (di)amide, more preferably optionally substituted alkoxy, cycloalkyl, amine, amide, carboxylic acid or optionally o-, m- or p- substituted phenyl wherein substituents include aryl, alkyl, cycloalkyl, heteroaryl or heteroalkyl, amine, amide, carboxyl, carbonyl etc, for example is cyclohexyl, cyclopentyl, ethoxy, $(CH_2)_2PhPh$, CH_2Ph, $CONH(CH_2)nCONH$, $CH_2CONH(CH_2)_2NH$, $CH_2PhNHCOCH_2$, $CH_2CH_2OCOCH_2$, succinimidyl ester, $NHCOCH_2$, $CH_2(CH_3)NCOCH_2$, $H_2N(CH_2)_2NHCOCH_2$, $H_2N(CH_2)_8NHCOCH_2$, $H_2NNHCOCH_2$, $CH_2CONH(CH_2)_2NHCOCH_2$, $HOPhCH_2N(CH_2CH_3.HOAc)(CH_2)_2NHCOCH_2$,

heterocyclic-$(CH_2)_4CONH(CH_2)_2NHCOCH_2$,
heterocyclic-NHCON(heterocyclic)$COCH_2$ and the like;

or Lig.a- is of the formula Lig.a^2-



wherein each of $C._{A1}$ and $C._{A2}$ is independently selected from aryl, heteroaryl, cyloalkyl and heterocyclic, more preferably from phenyl, or aryl containing 1 or 2 ring heteroatoms, or heterocyclic containing 1 ring heteroatom and/or 1 ring –C=C- group;

Each of up to seven R.a^5 is a substituent of a ring carbon or a ring heteroatom and:

is independently selected from H, halo, hydroxy, thiol, amine, COOH, hydrazine, cyano, saturated or unsaturated, substituted or unsubstituted $C_{1\text{-}20}$ branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, and wherein optional substituents are selected from any $C_{1\text{-}12}$ aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, such as =O, OCH_3, $CH_2Ph(OCH_3)_2$, $O(CH_2)_3CON(CH_3)$c.hex, $N(CH_2CH_2OH)_2$, c.hex, $COOCH_2CH_3$, CH_2CH_3;

or any two or more of R.a^5 form a one, two or three ring fused cyclic structure, preferably comprising a fused 3 ring aryl, 5-heterocyclic, 6-

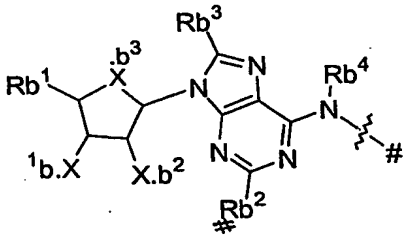
heterocyclic structure having 4 ring atoms common with the fused bicyclic Lig.a^2structure;

and R.a^6 is a moiety as defined for R.a^5 above;

and -L.a- is as hereinbefore defined for -L- and is suitably of formula -L.I- or -L.II- as hereinbefore defined, more preferably is selected from a single bond, amino acid or amide such as a peptide or polypeptide for example gly or gly_3, alkyl of formula $-(CH_2)_n$ where n is 3 to 8, preferably 3, 4 or 6, optionally including one or more heteroatoms or unsaturated groups, such as –O- or –S- or –CH=CH- and the like:

Lig.b is suitably of the formula Lig.b

Lig.b



wherein ring substituents X.b^1 and X.b^2 are independently selected from hydrocarbon such as alkyl or SR_X, $NR_{X.2}$ and OR_X wherein (each) R_X is selected from H, $C_{1\text{-}5}$alkyl, alkenyl;

ring heteroatom X.b^3 is selected from –S-, -O- and $-CH_2-$;

Rb^1 is selected from saturated or unsaturated, substituted or unsubstituted $C_{1\text{-}4}$ aliphatic, or $C_{1\text{-}3}$ alicyclic optionally including one or more heteroatoms N, O, S, P, wherein substituent(s) are selected from one or more cycloalkyl, heterocyclic, hydroxy,

oxo, halo, amine; preferably R.b^1 comprises a carbonyl substituted by H, alkyl or a linear or cyclic primary, secondary or tertiary amine, substituted C_{1-3} alkyl, cycloalkyl or amide, more preferably cyclopropyl, or $CONHC_{1-3}$alkyl such as CONHEt or CH_2OH

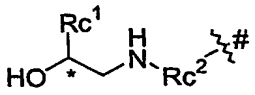
and each of R.b^2 and R.b^3 is selected from H, halo, hydroxy, thiol, amine, COOH, CHO, hydrazine, cyano or saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, preferably from H, halo or hydroxy, preferably H or Cl;

Rb^4 is H;

-L.b- is as hereinbefore defined for –L-, more preferably saturated and unsaturated substituted or unsubstituted C_{1-12} aliphatic or C_{1-24} aromatic as defined for –L- preferably including one or more heteroatoms O, S or N, cyclic or heterocyclic groups, more preferably is of formula -L.I- or -L.II- as hereinbefore defined, most preferably is –(CH_2)m wherein m is 2 to 12, preferably 3, 4, 6 or 8, or is –(Ph-$CH_2CONH)_2$ $(CH_2)_2$-;

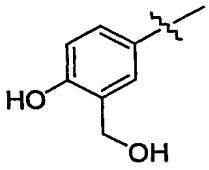
Lig.c is suitably a non-peptide of the formula

Lig.c HOC*(R.c^1)CH_2NH-R.c^2-



Where * indicates an optically active centre and

Wherein R.c^1 is C_{6-14} aryl optionally including one or more heteroatoms selected from H, O, optionally substituted by OH, Hal eg Cl, NH_2, NHC_{1-3}alkyl, sulphonamide, oxoamine (-$CONH_2$) and the like, more preferably mono, di or tri substituted phenyl or quinoline wherein substituents include OH, Cl or NH_2, more preferably m-CH_2OH, p-OH phenyl, m-,p-dihydroxy phenol or m-,m-dihydroxyphenol, m-,m-diCl, p-NH_2 phenol, p-OH, m-$CONH_2$ phenol or 5-OH, 8-quinoline and the like, such as



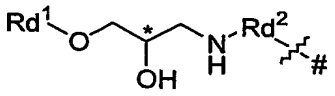
R.c^2 is selected from saturated or unsaturated, substituted or unsubstituted C_{1-20}, preferably C_{1-12}, branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any optionally substituted C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like and combinations thereof;

Preferably R.c^2 is selected from C_{1-6} branched or straight chain aliphatic, C_{6-10} araliphatic optionally substituted by OH and optionally including heteroatoms selected from N,O, preferably including an ether O, such as selected from $-(CH_2)_6OCH((CH_2)_3Ph)$, $CHCH_3(CH_2)_2Ph$, $CHCH_3CH_2PhOH$, $C(CH_3)_2CH_2$;

-L.c- is as hereinbefore defined for –L- and is suitably of formula -L.I- or -L.II- as hereinbefore defined, more preferably is selected from C_{1-12} alkyl, amide etc;

Lig.d is suitably a non-peptide of the formula

Lig.d R.d^1 $OCH_2C^*HOHCH_2NH$-R.d^2-#

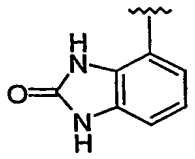


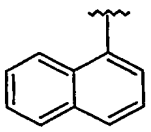
Where * indicates an optically active centre and where # indicates the site of linking to the fluorescent tagging moiety

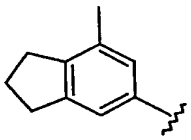
Wherein R.d^1 is saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more

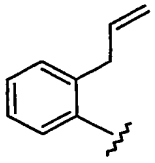
heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like;

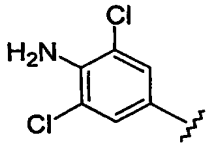
Preferably R.d[1] is substituted or unsubstituted C_{1-24} aralkyl or heteroaralkyl, including single ring and fused ring systems with (hetero)aryl or cycloalkyl rings, wherein optional substituents include C_{1-6} alkyl, alkoxy, ether, carbonyl, alkenyl, amine, amide each optionally carbonyl, amide, halo or OH subtitited, or halo such as chloro or OH, preferably R.d[1] is unsubstituted or substituted alkyl, alkenyl, halo, amine, amide, carbonyl, ketone, ether substituted phenyl or naphthyl, illustrated as follows, most preferably mono-, di-, tri- or tetra substituted mono or polycyclic fused aryl or cycloaryl or heterocycloaryl such as phenyl, carbazole or structures shown below or spiro ring systems, most preferably mono-, di-, tri- or tetra alkoxyalkyl, alkoxyalkoxyalkyl or CF_3 substituted phenyl or unsubstituted or monosubstituted naphthalene or 5,6 ring systems most preferably of the structures:





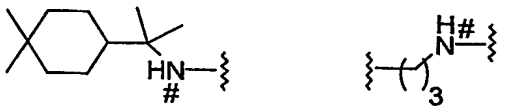






R.d[2] is substituted or unsubstituted amine, saturated or unsaturated, substituted or unsubstituted C_{1-12} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of

which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, more preferably amine, C_{1-6} branched or straight chain alkyl optionally including ether O, and optionally substituted by C_{6-10} aryl, for example of the formula:



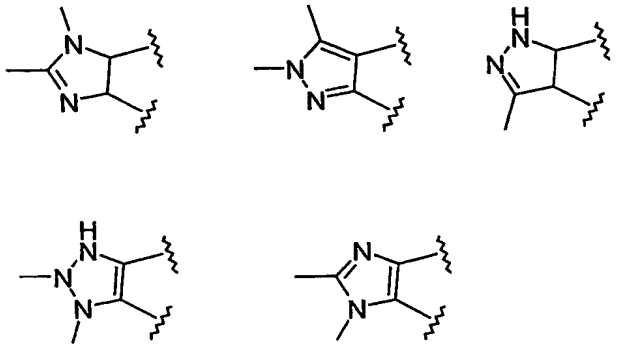
i.pr, i.bu, CH_2CH_2O (m-$CONH_2$, p-OH) phenol, CH_2CH_2O (o-OCH_3 phenol

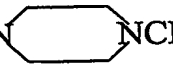
-L.d- is is as hereinbefore defined for –L- and is suitably of formula -L.I- or -L.II- as hereinbefore defined, more preferably is a single bond or is as hereinbefore defined for -L.a-;

Lig.e comprises a cell permeant moiety or is associated with a cell permeant L or Fl moiety and is suitably of the formula , in either of the following forms given:

Lig.e[1]

wherein h is selected from



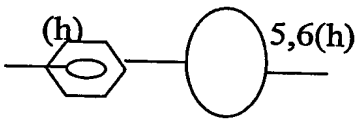
each optionally substituted by R.e^3 – R.e^4 wherein R.e^1 – R.e^4 are as R.a^1 – R.a^4 defined above or in which R.e^3 is C_{5-9}linear or branched alkyl, optionally mono or multi hydroxy or halo substituted or is aryl optionally substituted by alkoxy, sulfonyl and the like eg ortho-OEt, meta-SO_2N  NCH_3

each X is independently selected from H, =O, -OR.e^2, =N, HN, NR.e^5, HR.e^6 , and aryl optionally substituted by ether; or X is aryl optionally alkyl or alkoxy substituted such as Ph-ortho-$OCH_2CH_2CH_3$;

and where R.e^5 is as defined above for R.e^1 above or forms a fused cyclic ring together with the adjacent ring N atom; preferably 1 or 2 fused 5 membered cyclic rings;

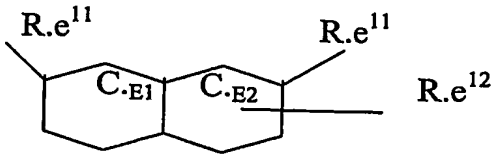
and $R.e^6$ is as defined above for $R.e^1$ above or is selected from optionally substituted phenyl wherein optional substituents include ether such as o-ethoxy or o-propoxy, alkyl, OH and the like, sulphonyl, carbonyl and the like substituted by heterocyclic, or cyclic C_{5-8} alkyl such as methyl, piperazinyl, sulphonyl and the like;

or Lig.e is of the formula $Lig.e^2$

$Lig.e^2$ 

Wherein each spiro ring optionally comprises zero or one or more heteroatoms h which are preferably N, more preferably  (h) comprises zero or 1 N heteroatom and  5,6(h) comprises zero, 1 or 2 N heteroatoms and is unsaturated or comprises one or two –C=C- or -C=N- groups;
and wherein each ring is optionally substituted by one or more oxo, CO, COOH, C_{1-6} alkyl or linear or cyclic alkoxy such as methoxy, ethoxy or cyclopentyloxy optionally substituted by one or more oxo, CO, COOH, CN, or C_{1-6} alicyclic or amine groups, amine or one or more spiro or fused heterocycles;

or Lig.e is of the formula $Lig.e^3$

$Lig.e^3$ 

Wherein each of $C._{E1}$ and $C._{E2}$ is independently selected from aryl, heteroaryl, cyloalkyl and heterocyclic, more preferably from phenyl, or aryl containing 1 or 2 ring heteroatoms, or heterocyclic containing 1 ring heteroatom and/or 1 ring –C=C- group;

Each of up to seven $R.e^{11}$ is a substituent of a ring carbon or a ring heteroatom and:

is independently selected from saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, and wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, such as =O, OCH_3, $CH_2Ph(OCH_3)_2$, $O(CH_2)_3CON(CH_3)$c.hex, $N(CH_2CH_2OH)_2$, c.hex, $COOCH_2CH_3$, CH_2CH_3;

or any two or more of $R.e^{11}$ form a one, two or three ring fused cyclic structure, preferably comprising a fused 3 ring aryl, 5-heterocyclic, 6-heterocyclic structure having 4 ring atoms common with the fused bicyclic $Lig.e^3$ structure;

and $R.e^{12}$ is a moiety as defined for $R.e^{11}$ above;

Preferably Lig.e is of the formula $Lig.e^1$ as hereinbefore defined in particular where $R.e^2$ and $R.e^3$ are respectively propyl and butyl;

-L.e- is suitably as hereinbefore defined for -L.a-.

Linking sites at which Lig-L- are linked to –Fl are suitably any sites which do not inhibit binding to receptor, more preferably are as indicated by # in the above formulae. Receptor binding is complex, and may require a specific binding site to be available and/or require a specific Ligand conformation.

The fluorescent ligands of the invention may be characterised by different linking sites linking ligand and fluorescent moiety. From a comprehensive knowledge of the binding behaviour and the specific binding sites, which remain unchanged in the fluorescent ligands of the invention, we have been able to determine suitable linking sites for desired retention of binding and pharmacological properties. Preferably the compounds of formula Lig-L-Tag include compounds representing all operative linking configurations exposing possible binding site options.

-Fl may include any red, green, near ir, blue or the like absorbing dyes and other classes of dyes. Suitably -Fl is selected from dyes in particular including fluorescein, fluorescein derivatives including FITC, and fluorescein-like molecules such as Oregon Green™ and its derivatives, Texas red™, 7-nitrobenz-2-oxa-1,3-diazole (NBD) and derivatives thereof, coumarin and derivatives, naphthalene including derivatives of dansyl chloride or its analogues or derivatives, Cascade Blue™, EvoBlue and fluorescent derivatives thereof, pyrenes and pyridyloxazole derivatives, the cyanine dyes, the dyomics (DY dyes and ATTO dyes) and fluorescent derivatives thereof, the Alexafluor dyes and derivatives, BDI dyes including the comercially available Bodipy™ dyes, erythosin, eosin, pyrenes, anthracenes, acridines, fluorescent phycobiliproteins and their conjugates and fluoresceinated microbeads, Rhodamine and fluorescent derivatives thereof including Rhodamine Green™ including the tetramethylrhodamines, X-rhodamines and Texas Red derivatives, and Rhodol Green™, coupled to amine groups using

the isocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups and other red, blue or green absorbing fluorescent dyes in particular red absorbing dyes as reviewed in Buschmann V et al, Bioconjugate Chemistry (2002), ASAP article.

More preferably -Fl is selected from fluorescein derivatives and fluorescein-like molecules such as Oregon Green™ and its derivatives, Texas red™, 7-nitrobenz-2-oxa-1,3-diazole (NBD) and derivatives thereof, coumarin and derivatives, naphthalene including derivatives of dansyl chloride or its analogues or derivatives, Cascade Blue™, EvoBlue and fluorescent derivatives thereof, pyrenes and pyridyloxazole derivatives, the cyanine dyes, the dionics (DY dyes and ATTO dyes) and fluorescent derivatives thereof, the Alexafluor dyes and derivatives, BDI dyes including the commercially available Bodipy™ dyes, erythosin, eosin, FITC, pyrenes, anthracenes, acridines, fluorescent phycobiliproteins and their conjugates and fluoresceinated microbeads, Rhodamine derivatives thereof including Rhodamine Green™ including the tetramethylrhodamines, X-rhodamines and Texas Red derivatives, and Rhodol Green™.

More preferably -Fl comprises fluorescein, Texas Red ™, Cy5.5 or Cy5 or analogues thereof, BODIPY ™ 630/650 and analogues thereof, DY-630, DY-640, DY-650 or DY-655 or analogues thereof, ATTO 655 or ATTO 680 or analogues thereof, EvoBlue 30 or analogues thereof, Alexa 647 or analogues thereof.

Suitably -Fl is derived from any of the above commercially available fluorophores, comprising or modified to comprise a reactive group facilitating linking to a ligand as hereinbefore defined. Preferably Fl comprises any of the above commercially available fluorophores modified to form a derivative or

group of derivatives suitable for visualising ligand binding in a kit as hereinbefore defined preferably wherein Fl- is Fl'-t- wherein –t- comprises functionality derived linking to a precursor ligand as hereinbefore defined and may optionally comprise a proximal unsaturated or aryl moiety, comprising a medial short, medium or long chain alkynyl or cycloalkyl moiety and comprising a moiety derived from linking via a reactive group as hereinbefore defined such as carboxyl, sulphonate or as a heteroatom such as O or S or methylene derived from linking at an alkylhalide such as methylbromide, haloacetamide, sulphonate ester or the like electrophilic group.

For example -Fl may include a substituent –t- which is a heteroaryl or alkenyl such as mono-, di- or tri -enyl group which shifts the fluorescence of the compound to the red part of the spectrum and raises the absorption max value as in US 5187288; or may include alkenyl substituent linked to one or more of an aryl, carbonyl or like group, preferably linked to a fatty acid sidechain comprising $(CH_2)nCO_2H$ where n = 5 – 22 as in US 5330854, more preferably linked via an aryloxymethylene to a and carbonyl; or may include an aryl alkenyl aryl group as in US 6005113.

Preferred Bodipy ™ (4,4-difluoro-4-bora-3a,4a-diaz-s-indacene) fluorophores include those which span the visible spectrum (U.S. Pat. No. 4,774,339; U.S. Pat. No. 5,187,288; U.S. Pat. No. 5,248,782; U.S. Pat. No. 5,274,113; U.S. Pat. No. 5,433,896; U.S. Pat. No. 5,451,663). A preferred member of this group is selected from any heteroaryl substituted BODIPY ™ dyes as described in the above patents the contents of which are incorporated herein by reference.

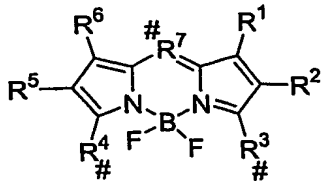
Suitably -Fl comprising a BODIPY ™ structure is characterised by a dipyrromethene boron difluoride core, optionally modified by one or two fused

rings, optionally substituted by one or several substituents such as alkyl, alkoxy, aryl, heterocyclic and the like, wherein one substituent –t- is adapted for linking as hereinbefore defined to a ligand precursor as hereinbefore defined, the substituent –t- optionally comprising a proximal unsaturated or aryl moiety, comprising a medial short, medium or long chain alkynyl or cycloalkyl moiety and comprising a moiety derived from linking via a reactive group as hereinbefore defined such as carboxyl, sulphonate or as a heteroatom such as O or S or methylene derived from linking at an alkylhalide such as methylbromide, haloacetamide, sulphonate ester or the like electrophilic group.

For example -Fl may include a substituent –t- which is heteroaryl or alkenyl such as mono-, di- or tri -enyl group which shifts the fluorescence of the compound to the red part of the spectrum and raises the absorption max value as in US 5187288; or may include alkenyl substituent linked to one or more of an aryl, carbonyl or like group, preferably linked to a fatty acid sidechain comprising $(CH_2)nCO_2H$ where n = 5 – 22 as in US 5330854, more preferably linked via an aryloxymethylene to a and carbonyl; or may include an aryl alkenyl aryl group as in US 6005113.

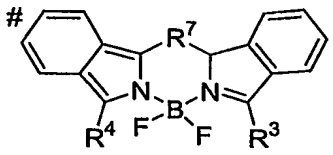
More preferably -Fl is of the formula -Fl^1:

Fl^1 dipyrromethene borondifluoride analogues



Wherein R7 is N or C-R8;

Substituents R1, R2, R3, R4, R5, R6 and R8 which may be the same or different are H, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, or acyl wherein the alkyl portions of each contain fewer than 20 carbons; or substituted or unsubstituted aryl or heteroaryl; preferably at least four of R^1 to R^8 are non-hydrogen, alternatively adjacent substituents R1 and R2 taken in combination and adjacent substituents R5 and R6 taken in combination form fused 6-membered (hetero) aromatic rings

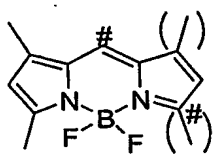


wherein each fused ring is optionally and independently substituted by H, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, alkylthio, alkylamido, amino, (mono or dialkyl)amino (wherein the alkyl portions of each contain fewer than 20 carbons), or substituted or unsubstituted aryl, heteroaryl, arylamido, heteroarylamido, aryloxy, heteroaryloxy, arylamino or heteroarylamino; or 1 to 2 additional fused benzo or heteroaromatic rings that are optionally substituted or unsubstituted.

Preferably any or all of $R^{2,3}$ to $R^{4,5}$ is heteroaryl, more preferably a single ring single heteroatom such as such as pyrrole, thiophene, furan or single ring di heteroatom structure such as oxazole, isoxazole, oxadiazole, imidazole, or

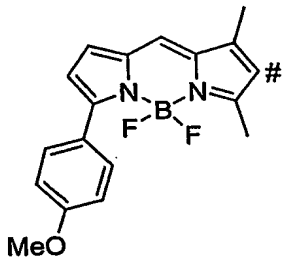
multi ring such as benzoxazole, benzothiazole, benzimidazole, or multi ring one heteroatom structure such as benzofuran, indole, preferably thienyl.

More preferably Fl is selected from the BODIPY core structures of formulae FL.A1 or FL.A2 as shown below, in each case = indicating the attachment site of a sidechain and # indicating the linking site to Lig in the compound of formula I:

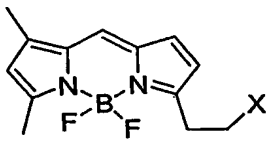


Fl.A1

preferably comprising or derived from BODIPY TMR or BODIPY FL (4,4-difluoro-5,7dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid) or BODIPY FL ethylene diamine

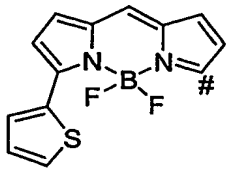


BODIPY TMR

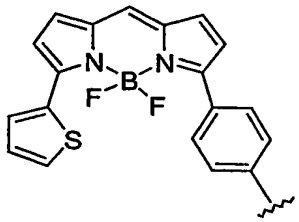


BODIPY FL ethylene diamine (X is $CONH(CH_2)_2NH_2$) or BODIPY FL (X is COOH)

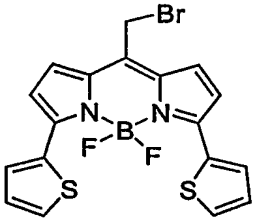
Or Fl.A2

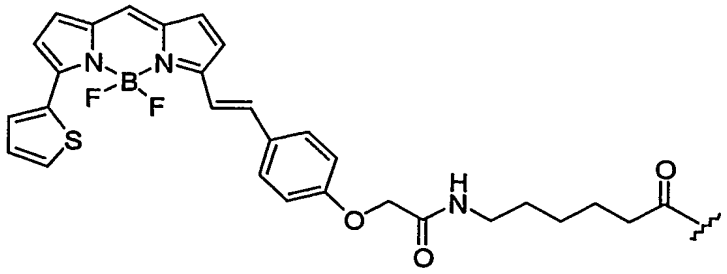


preferably comprising or derived from BODIPY 630/650 or BODIPY 630/650 methyl bromide



BODIPY 630/650 methyl bromide





most preferably the succinimidyl esters thereof.

In a further aspect of the invention there is provided a process for the preparation of a kit as hereinbefore defined comprising the reaction of one or each of a plurality of ligand precursors of formula

Lig-L-Z_L

comprising a reactive site wherein $-Z_L$ is a leaving group

with a plurality of analytical tagging substrates of formula

Tag-Z_T

comprising a reactive site wherein -Z_T is a leaving group;

wherein the or each Lig-L-Z_L is capable of reaction with a plurality of Tag-Z_T, to form a plurality of compounds Lig-L-Tag.

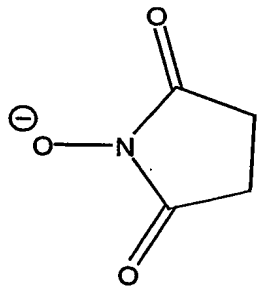
Preferably some or each Tag-Z_T is an entity Fl-Z_F comprising a reactive site wherein Z_F is a leaving group as hereinbefore defined and comprises any known or novel fluorophore, whereby the process is a process for preparing a kit comprising a plurality of compounds of which one or more or all of which are of formula Lig-L-Fl.

Suitably Ligand Lig-L-Z_L and fluorophore Fl-Z_F have suitable reactive end group functionalities for linking, as hereinbefore defined, for example selected from combinations of electrophilic (Lig-L-Z_L or Fl-Z_F usually Fl-Z_F) and nucleophilic (Lig-L-Z_L or Fl-Z_F usually Lig-L-Z_L) groups such as:

Fl-Z_F	Lig-L-Z_L	Lig-L-Fl	-Z_F	-Z_L
Electrophilic	Nucleophilic	Resulting covalent linkage	leaving gp	leaving gp
Carboxylic acid	alcohol	ester	-OH	-H
Carboxylic acid	amine	carboxamide	-OH	-H
Carboxylic acid	hydrazine	hydrazide	-OH	-H

	Alkyl halide	alcohol	ether	-Hal	-H
	Alkyl halide	thiol	thioether	-Hal	-H
	Alkyl halide	amine	alkylamine	-Hal	-H
	Alkyl halide	COOH	ester	-Hal	-H
	Haloacetamides	thiols	thioethers	-Hal	-H
	Sulphonate esters	amines	alkyl amines	RSO_3-	-H
	Sulphonate esters	alcohols	ethers	RSO_3-	-H
	Sulphonate esters	thiols	thioethers	RSO_3-	-H
	Sulphonyl halides	amines	sulphonamides	-Hal	-H
	Sulphonyl halides	alcohols	sulphonate esters	-Hal	-H
	Succinimide ester	alcohols	esters	-OSu*	-H
	Succinimide ester	alkoxides	esters	-OSu*	-H or M^+
	Succinimide ester	thiols	thioesters	-OSu*	-H
	Succinimide ester	amine	carboxamide	-OSu*	-H
	Succinimide ester	hydrazine	hydrazide	-OSu*	-H

Wherein * is



Preferably Lig-L-Z_L comprises no protecting group and is capable of reaction with Tag-Z_T without degradation of functionality by choice of reaction and of respective reactive sites; or Lig-L-Z_L comprises one or more protecting groups which are adapted for removal under ambient conditions, for example under neutral pH, room temperature or the like.

In a particular advantage of the present invention linker –L- facilitates linking of fluorescent moiety and ligand, in cases that end groups of respective moieties are not reactive, or that stereochemistry or other effects inhibit linking, or that reaction of existing reactive groups in commercially available compounds Lig-L-Z_L and Tag-Z_T would require the inclusion of protecting groups for functionalities present in Lig-L- and Tag-, in which case a linker is usually a difunctional short, medium or long chain structure. In a further advantage of the invention linker –L- may confer properties facilitating crossing the cell membrane, hydrophobicity, hydrophilicity and the like as required, in which case a linker is usually any functionalised structure.

Preferably – LZ_L is selected from H, halo, hydroxy, thiol, amine, COOH, hydrazine, cyano and saturated; or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, and comprising a reactive group or site for linking to fluorophore selected from hydroxy, alkoxy, thiol, thioxy, amine, hydrazine, carbonyl and the like. In the case that – LZ_L comprises H, then a reactive site is usually present on the ligand moiety, whereby Lig-Z_L is reactive with Tag – Z_T.

Preferably Z_L comprises a leaving group selected from H, OH, SH, amine, alkyl, and the like.

More preferably –L-Z_L is selected from H, hydroxy, thiol, amine; and C_{1-12} alkyl, alkenyl, alkynyl, alkoxy, amino, cycloalkyl, heterocyclic, aryl, heteroaryl, and combinations thereof such as aralkyl, wherein heteroatoms are as hereinbefore defined, optionally substituted as hereinbefore defined wherein substituents are selected from and C_{1-6} aliphatic, aromatic or alicyclic substituents as defined, hydroxy, thiol, halo, amine, oxo, and the like.

Suitably – L-Z_L is characterised by a short, medium or long chain moiety, and functionality derived from its synthesis by reacting a linker precursor and a ligand precursor comprises amino, alkoxy, alkthioxy, oxo, amide and the like, and a reactive end group for reaction with Tag-Z_T may comprise amine, hydroxy, thiol, carboxylic acid groups and the like, as hereinbefore defined, whereby -L-Z_L may for example comprise an amino alkylthiol, amino alcohol, a hydroxy alkyl carboxylic acid, hydroxy alkylamine and the like. Preferably –L-Z_L is selected from mono or diamino menthane, mono or diamino ethane, mono or dithio ethane, mono or dihydroxy ethane, amino acid or polypeptide, or from mono or polyether diamine or dithiol such as mono or polyethylene glycoldiamine or dithiol.

Preferably a reactive linker –L-Z_L retaining pharmacological activity comprises a difunctional linear or cyclic alkyl of formula -L- Z_L.I

-L- Z_L.I

$$-Y_L'\left[(CR_L)q_L\ A\right]q_L''\ (CR_L)q_L'\ Y_L\ Z_L$$

wherein -Y_L'- is selected from a single bond, methylene, alkyne, alkene, NH, NR, O, NRCO, S, CO, NCO, CHHal, P and the like wherein Hal is any halogen selected from chlorine, iodine, fluorine;

$-Y_LZ_L$ is a reactive group as hereinbefore defined, wherein Z_L is a leaving group as hereinbefore defined such as H, OH, SH, halogen, amine, aliphatic and the like;

-A- is a property conferring group selected from –O-, -C(=O)- cycloalkyl, heterocyclic, alkenyl, aryl, heteroaryl as hereinbefore defined and combinations thereof and the like;

q_L and q_L' are independently zero or are selected from a whole number integer from 1 to 10, preferably the sum of q_L and q_L' is zero, 1 or greater than or equal to 2 and less than or equal to 12; and

q_L'' is 0 or 1 to 30, or indicates a polymeric repeat unit of 30 up to 300

and each R_L is selected independently from H, C_{1-3} alkyl, C_{1-5} alkoxy and the like.

Preferably -L-Z_L is of formula -L-Z_L.II

-L-Z_L.II $\quad -Y_L'[(CH_2)_2O]q_L''(CH_2)_2Y_LZ_L$

wherein Y_L', q_L'' and Y_LZ_L are as hereinbefore defined.

In a further aspect of the invention there is provided a known or novel compound Lig-L-Fl as hereinbefore defined wherein the compound is associated with information relating to its pharmacological properties in the form of Spectral Properties given as Excitation Max and Emission Max, Fluorescence Lifetime and Emission quantum yield and Pharmacology defined in terms of cells expressing a biogenic amine GPCR receptor as hereinbefore defined or expressing an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases, and given as the Inhibition or Antagonism of receptor binding or of receptor functionality together with a value for the Inhibition (pK_B) or Antagonism (pK_I), and optionally together

with images of the pharmacological binding illustrating the defined inhibition or antagonism.

Preferably the compound is associated with information relating to its pharmacological properties wherein pharmacology is defined in terms of a cell or protein wherein the cell comprises a GPCR or the protein is a G-protein, preferably in terms of a CHO cell comprising GPCR receptors selected from adenosine A_1-, A_{2A}-, A_{2B}- and A_3-receptors, β_1, β_2- and β_3- adrenoceptors, or comprises an inhibitor of an intracellular enzyme such as cyclic nucleotide phosphodiesterases, more preferably in terms of CHO-cells expressing human adenosine A_1-receptor or beta-adrenoceptor or an inhibitor of an intracellular enzyme such as an inhibitor of intracellular enzymes PDE 1-5 and which is given as the Inhibition of ^{3}H-DPCPX binding (membranes or whole cells) (pK_B) and /or Antagonism of NECA-stimulated cAMP accumulation (pK_I), Antagonism of NECA-stimulated inositol phosphate accumulation (pK_I), and/or the Stimulation of cellular responses such as subcellular responses (EC_{50}) and/or the Inhibition of cyclic nucleotide phosphodiesterase activity (pK_B).

In a further aspect of the invention there is provided a novel compound of the formula

Lig – L - Fl

Wherein Lig- comprises a biogenic amine GPCR receptor ligand selected from a) an adenosine receptor antagonist b) an adenosine receptor agonist c) a beta-adrenoceptor agonist and d) a beta-adrenoceptor antagonist; or Lig- comprises e) an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases; or a derivative or analogue thereof;

-L- is a single bond or is any linking moiety which may be monomeric, oligomeric having oligomeric repeat of 2 to 30 or polymeric having polymeric repeat in excess of 30 up to 300;

and Fl is any known or novel fluorophore, preferably as hereinbefore defined;

with the proviso that:

a) when Lig is XAC and L is a single bond (ie n=0) or L is gly and n=3, Fl is not fluorescein; or

when Lig is XAC and L is a single bond and n=1, Fl is not fluorescein or NBD;

b) when Lig is ADAC and L is a single bond, Fl is not fluorescein, NBD or Rhodamine; or

when Lig is NECA (incorporating the moiety $-(CH_2)m$) and L is a single bond, when m is 2,4,6,8 or 10 then Fl is not NBD, or when m is 3,4,6,8,10 or 12 then Fl is not dansyl; or

c) -

d) when Lig is CGP12177 and L is mono amine menthane, Fl is not BODIPY® TMR; or

when Lig is CGP12177 and L is 1,1,4,4-tetramethyl butylamine, Fl is not BODIPY® FL, FITC, eosin or erythosin; or

when Lig is CGP12177 and L is a single bond, Fl is not NBD; or

when Lig is alprenolol and L is $-C(CH_3)_2-$ or a single bond, Fl is not NBD.

Optionally additionally a) when Lig is XAC and L is a single bond Fl is not BODIPY ™ 630/650; or

b) when Lig is ABEA and L is a single bond Fl is not BODIPY™ 630/650

Preferably a ligand or fluorescent ligand of the invention is an agonist which maintains its binding affinity and its functional activity or is an antagonist which maintains its binding affinity on linking or when linked to fluorescent moiety Fl. Fluorescent ligands may have affinity such that they bind permanently, semi-permanently or transiently, ie may retain bound or may be washed away when unbound ligand is washed away.

Fluorescent ligands of the invention may be inherently optically active or may be functionalised, in known manner, to be optically active, and any such ligand may be present as a racemate or as one of its optically active isomers.

More preferably the fluorescent ligand Lig – L - Fl of the invention is selected from formulae Lig.aL.a-Fl.a – Lig.eL.eFl.e:

Lig.a-L.a – Fl.a

wherein Lig.a comprises xanthine like structures including XAC, theophyline, caffeine, theobromine, dyphilline, enprofylline and the like; or fused biaryl structures including papaverine, cilostamide and the like; and analogues thereof;

Fl is BODIPY 630/650 and the moieties are directly or indirectly covalently linked via the XAC-8-substituent which preferably terminates with a diamino moiety;

Lig.b- L.b – Fl.b

wherein Lig.b comprises adenosine like structures including ADAC, ABEA, NECA and analogues thereof;

and Fl = BODIPY 630/650 and L.b is preferably a diamino or biphenylamide as hereinbefore defined;

Lig.c- L.c – Fl.c

wherein Lig.c comprises ethanolamine structures including Salmeterol, salbutamol, terbutaline, quinprenaline, bambuterol, fenoterol, reprotolol, tulobuterol, clenbuterol and analogues thereof; L.c is as hereinbefore defined and Fl is selected from BODIPY 630/650);

Lig.d- L.d– Fl.d

wherein Lig.d comprises oxypropanolamine structures including CGP12177, propranolol, practolol, acebutalol, betaxolol, ICI 118551, alprenolol, celiprolol (celectol), metoprolol (betaloc), CGP20712A, atenolol, bisoprolol, misaprolol, carvedilol, bucindolol, esmolol, labetalol, nadolol, nebivolol, oxprenolol, xamoterol, pindolol, sotalol, timolol and analogues thereof; and

L.d is cyclic (di)amine or linear (di)amine as hereinbefore defined and

Fl = BODIPY 630/650;

Lig.e- L.e– Fl.e

wherein Lig.e comprises xanthine like structures including XAC, theophylline, caffeine, theobromine, dyphilline, enprofylline, sildenafil, EHNA (erythro-9-(2-hydroxyl-3-nonyl)adenine), zaprinast and the like; or spiro bicyclic structures including bypyridines such as amrinone, imidazolines such as CI930, dihydropyridazinones such as indolan, rolipram, SB207499, and the like; or fused biaryl structures including papaverine, dihydroquinilones such as cilostamide, dipyridamole, vinpocetine and the like and analogues thereof; L.e is as hereinbefore defined;

and Fl = BODIPY 630/650.

More preferably a novel compound of the formula Lig-L-Fl comprises a moiety Lig.a-L.a- to Lig.e-L.e- as hereinbefore defined

with the proviso thatin Lig.a^1 -L.a^1- when each of R.a^1 and R.a^2 is propyl, R.a^3 is H and R.a^4 is –Ph-$OCH_2CONH(CH_2)_2NH$-, and L is a single bond or gly_3 or NCS then Fl is not fluorescein; or

when each of R.a^1 and R.a^2 is propyl, R.a^3 is H and R.a^4 is –Ph-$OCH_2CONH(CH_2)_2NH$-, and L.a is NCS then Fl is not NBD; or

or with the proviso that in Lig.b-L.b-

When R.b^1 is CH_2OH, R.b^2 and R.b^3 are H and L is –(Ph-$CH_2CONH)_2$ $(CH_2)_2$- then Fl is not fluorescein, NBD or Rhodamine; or

When R.b^1 is CONHEt, R.b^2 and R.b^3 are H and L is –(CH_2)m where m is 2,4,6,8,10 then Fl is not NBD or where m is 3,4,6,8,10,12 then Fl is not dansyl;

Or with the proviso that in Lig.d-L.d when Lig.d is CGP12177 and R.d^2 is monoamine menthane, then Fl is not BODIPY ® TMR or when R.d^2 is – $C(CH_3)_2(CH_2)_2C(CH_3)_2NH$- then Fl is not BODIPY ® FL or when R.d^2 is – $C(CH_3)_2(CH_2)_2C(CH_3)_2NHCSNH$- then Fl is not FITC, eosin or erythosin, or

When R.d^1 is o-prop-2-enyl phenyl and R.d^2 is $C(CH_3)_2$ then Fl is not NBD.

Optionally additionally in a) when each of R.a^1 and R.a^2 is propyl, R.a^3 is H and R.a^4 is –Ph-$OCH_2CONH(CH_2)_2NH$-, and L.a is a single bond then Fl is not BODIPY ® 630/650; or in

b) where m is 4 then Fl is not BODIPY ® 630/650

In a further aspect of the invention there is provided a process for the preparation of tagged ligands, suitably fluorescently tagged ligands of formula Lig-L-Tag suitably Lig-L-Fl as hereinbefore defined comprising: reacting reactive ligand Lig-L-Z_L with reactive tag Tag-Z_T, suitably reactive fluorophore Fl-Z_F comprising a reactive group having leaving group Z_F or Z_T wherein reactive groups are selected so as to enable reaction with a fully deprotected ligand ie without the need for protecting groups or so as to enable reaction with protecting groups present which may be removed under mild

conditions.

Preferably reactive groups Z_L and Z_F or Z_T are selected so as to enable reaction with a fully deprotected ligand, for example Z_L comprises amine or alcohol or thiol and Z_T or Z_F comprises succinimide ester.

Alternatively a protecting group which may be removed under mild conditions comprises benzyloxycarbonyl and the like which are removed at ambient conditions such as room temperature or under conditions which do not prejudice functional groups such as the glycosidic group in Lig.b.

The process of the invention is characterised by a high yield of tagged ligand Lig-Ln-Tag suitably fluorescent ligand Lig-Ln-Fl as hereinbefore defined by use of chemoselectivity and is superior to known methods which prejudice yields by use of non chemoselective reactive groups or protecting groups.

Preferably the process comprises preparing the ligand, by routes as known in the art, modified for the introduction of linker -L-Z_L where present, and linking to fluorophore by reaction of reactive groups, for example of amine on the ligand Lig or linker L, with carbonyl on the fluorophore Fl-Z_F.

Preferably the compounds Lig-Ln-Fl of the invention are obtained by:
a), b), c), d), e) reacting the unprotected primary alkyl amine group of Lig.a-L.a-Z_L.a, Lig.b-L.b-Z_L.b, Lig.c-L.c-Z_L.c, Lig.d-L.d-Z_L.d or Lig.e-L.e-Z_L.e as hereinbefore defined with Fl-Z_F comprising a reactive succinimidyl ester group in solvent at ambient temperature without need for subsequent deprotection. In a particular advantage of the invention the method provides greater yield than with the prior art processes.

In a further aspect of the invention there is provided a process for the preparation of a reactive ligand of formula Lig-L-$Z_L$as hereinbefore defined comprising: obtaining where commercially available or preparing the ligand Lig-, by routes as known in the art, and introducing linker -L-Z_L and removing any protecting group present during the synthesis, optionally replacing with a protecting group which may be removed under ambient conditions.

Preferably the process comprises:

a), e) ring closure of 5,6-diamino-1,3-dialkyl uracil with the appropriate substituted aldehyde under acid conditions with ferric chloride, or obtaining compounds by the process of Jacobsen *et al* as hereinbefore referred; or as referred in publications described by Galatin (Viagra) http://views.vcu.edu/~pgalatin/viagra.html;

b) reacting Lig.b- comprising a protected inosine derivative with chlorinating agent and linking the chloro derivative with the amine group of a suitably protected amine reactive linker H–L-P_L wherein P_L comprises *N*-benzyloxycarbonyl- to form Lig-L-P_L and removing P_L to generate Lig-L-Z_L; preferably R.b^1 comprises a OH terminating group and protected inosine comprises Acyl protecting groups or R.b^1 comprises a stable group such as amine or amide and protected inosine comprises 2,2-dimethoxypropane protecting group; preferably the protected inosine is reacted with oxidising agent and protected alkylamine which is an *N*-alkylcarboxamide with removal of amine protecting group to generate a reactive ligand;

c), d) reacting m-hydroxybenzaldehyde with dioxane to form a protected diol, converting aldehyde to epoxy, reacting a suitably protected linker such as Boc-L.c-H which opens up the epoxy and supplies Lig-L-P_L and deprotecting under acid conditions.

In a further aspect of the invention there is provided a novel reactive ligand Lig-L-Z_L useful for linking to any suitable tag Tag-Z_T, in particular any suitable fluorophore Fl-Z_F as hereinbefore defined, wherein Lig-, -L- and –Z_L and Tag, Fl, Z_T and Z_F are as hereinbefore defined,

with the proviso that when Lig is Lig.a as hereinbefore defined and Z_L is -CO_2H, R.a^1 and R.a^2 are CH_3 or n-C_3H_7, then R.a^4 is not H, CH_2CO_2H, or $CH_2CONHBzCH_3$; or when Z_L is OH and R.a^1 and R.a^2 are n-C_3H_7, then R.a^4 is not $CH_2CONHBz$; or when Z_L is NH_2 and R.a^1 and R.a^2 are n-C_3H_7, then R.a^4 is not CH_2CO-, CH_2CONH or compounds as disclosed in Jacobsen *et al* as hereinbefore referred.

Preferably a linker precursor used in the process for preparing a compound Lig-L-Z_L and for preparing a compound Lig-L-Fl retaining pharmacological activity according to the present invention comprises a difunctional linear or cyclic alkyl comprising a moiety –L-Z_L as hereinbefore defined, of formula Y_L 'Z_L'-L- Y_L Z_L.

Preferably a linker precursor Y_L 'Z_L'-L- Y_L Z_L is selected from any C_{1-12} substituted or unsubstituted alkylamine, aminoacid, cycloalkyl, aryl, heteroaryl, aralkyl, and the like providing a reactive end group for linking to Fl, more preferably selected from (di)amine, comprising cyclic or linear amine, more preferably diamine menthane, or diamino ethylene, amino acid or polypeptide, or from mono or polyether diamine such as polyethylene glycoldiamine, more preferably from $H_2N(CH_2)_4NHCO_2CH_2Ph$, $H_2N(CH_2)_5NHCO_2CH_2Ph$, $H_2N((CH_2)_2O)_2(CH_2)_2NHCO_2CH_2Ph$ and $H_2N(CH_2)_nNHBoc$ where n is 2 to 8.

More preferably a linker precursor comprises a compound of formula

$Z_L'Y_L'$ $(CR_{L.2})q_L$ A q_L'' $(CR_{L.2})q_L'$ Y_L Z_L

wherein $Z_L'Y_L$ and Y_L Z_L are independently selected from H, CO_2H, NH2, O, P, and groups providing on reaction a single bond, alkyl such as methylene, alkyne, alkene, NH, NR, O, NRCO, S, CO, NCO, CHHal, P and the like wherein Hal is any halogen selected from chlorine, iodine, fluorine, or

wherein Y_L Z_L is selected from protecting leaving groups such as -$NHCO_2CH_2Ph$;

$-Y_LZ_L$ is a reactive group as hereinbefore defined, wherein Z_L is a leaving group as hereinbefore defined, or a protecting leaving group such as H, OH, SH, halogen, amine, aliphatic, N-alkylcarboxamide, Boc and the like;

-A- is a property conferring group selected from –O-, cycloalkyl, alkenyl, aryl and combinations thereof and the like;

q_L and q_L' are independently zero or are selected from a whole number integer from 1 to 10, preferably the sum of q_L and q_L' is zero, 1 or greater than or equal to 2 and less than or equal to 12; and

q_L'' is 0 or 1 to 30, or indicates a polymeric repeat unit of 30 up to 300

and each R_L is selected independently from H, C_{1-3} alkyl, C_{1-5}alkoxy and the like.

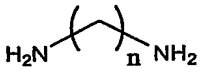
Preferably a linker precursor is of formula Y_L 'Z_L '$[(CH_2)_2O]q_L''(CH_2)_2$ Y_L Z_L

A linker precursor may be commercially available or may be prepared by known means. A linker may be installed as an independent entity or may be

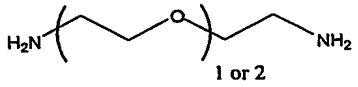
constructed as part of a synthetic process using techniques as known in the art, preferably is synthesised as an additional substituent on the ligand moiety or fluorescent moiety.

Most preferably a linker precursor is a mono, di or mixed amine, hydroxy, thiol, carbxylic acid, acid chloride, acid fluoride, acid bromide, (acid halide), isocyanate NCO, isothiocyanate NCS, halide, alkylhalide, aldehyde, epoxide, sulphonyl chloride SO_2Cl or hydrazine $NHNH_2$,

for example a diamine of formula

Diaminoalkyl 

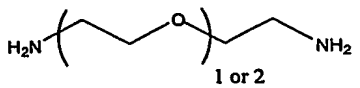
n = 2-12

Or Diamino-PEG ether 

or $H_2N(CH_2CH_2O)pCH_2CH_2NH_2$

wherein p is a whole number integer selected from 1 or 2 to 30, eg 1 or 2 to 20, or represents a polymeric repeat of the order of 30 up to 300 preferably 100 up to 300.

In a further aspect of the invention there is provided a novel linker for linking any known biogenic amine G protein coupled receptor ligand and fluorophore comprising an ether preferably a PEG ether as hereinbefore defined, and retaining pharmacological activity, more preferably of formula

Diamino-PEG ether 

or $H_2N(CH_2CH_2O)pCH_2CH_2NH_2$

wherein p is a whole number integer selected from 1 or 2 to 30, eg 1 or 2 to 20, or represents a polymeric repeat of the order of 30 up to 300.

In a further aspect of the invention there is provided a novel fluorophore of formula Fl-Z_F or Fl-t-Z_F useful for linking to any suitable ligand of formula Lig-L-Z_L for use in the present invention,
wherein Fl, Z_F, -t-, Lig, L and Z_L are as hereinbefore defined, preferably wherein –t- comprises -(PEG ether)n where n= 1 or 2 to 30, or 30 to 300 as hereinbefore defined.

The fluorescent ligands of the invention are useful for visualising receptors, assessing pharmacological properties of the fluorescent ligand but also in other applications such as high throughput screening of novel chemical entities that bind to the target receptor using confocal microscopy or fluorescence correlation spectroscopy.

In a further aspect of the invention there is provided a method for cell binding and visualisation comprising contacting a fluorescent ligand as hereinbefore defined with a sample in manner to facilitate binding thereof, and detecting changes in fluorescence.

A sample may comprise cell material, selected from cells, cell extracts, cell homogenates, purified or reconstituted proteins, recombinant proteins or synthesised proteins and the like. Samples comprising cell material may be derived from plants, animals, fungi, protists, bacteria, archae or cell lines derived from such organisms. Animal or plant cells used to prepare the sample may be healthy or disfunctional and are optionally used in the diagnosis of a disease such as leukaemia or cancer. In a preferred embodiment of the

invention the sample comprises mammalian cells, extracts and homogenates thereof.

In a preferred embodiment the cell or protein is a cell comprising a GPCR or the protein is a G-protein. More preferably the sample comprises GPCR receptors selected from adenosine A_1-, A_{2A}-, A_{2B}- and A_3-receptors, β_1, β_2- and β_3- adrenoceptors, or comprises inhibitors of intracellular enzymes such as cyclic nucleotide phosphodiesterases, most preferably CHO-cells expressing human adenosine A_1-receptor or beta-adrenoceptor or an inhibitor of an intracellular enzyme such as an inhibitor of intracellular enzymes PDE 1-5.

Receptors may be provided in membrane samples or in acutely dispersed cell samples, for example endogenous receptors such as A_1-AR in acutely dispersed cells. The adenosine receptor binding site is located deep within the pocket of the receptor, whereby a fluorescent ligand with linker is a preferred fluorescent (ant)agonist. Whilst there is considerable freedom in modifying the ligand and retaining antagonist binding activity, it is harder to retain agonist activating activity, ie activating the receptors functions on binding.

Preferably the method includes detecting a change in fluorescence such as the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarisation or a combination thereof. The optical response is detected by known means such as cameras, film, laser-scanning devices, fluorometers, photodiodes, quantum counters, microplate, microscopes, fluorescent microscopes such as epifluorescence or confocal, cytometers, readers and the like. Where the sample is examined using a flow cytometer, examination of the sample optionally includes sorting components of the sample according to their fluorescence response.

A method for binding and detection according to the invention may be *in vitro* or *in vivo*.

In a particular advantage of the invention the novel fluorescent ligands are suitable for use in combination with FCS enabling the study of ligand-receptor binding at the single molecule level. Because of the nature of the events being monitored FCS is ideal for the study of thermodynamic and kinetic features of molecular interactions in solution. Another particular advantage of the invention is that the FCS approach can be adapted to monitor ligand-receptor binding at the single molecule level using photon counting fluorescence intensity measurements. This removes any requirement for the molecules to be moving within the confocal volume.

With ligands showing low background fluorescence it is not necessary to remove unbound ligand by washing before performing either confocal microscopy or FCS. It is therefore possible to measure fluorescence with time, in both time and concentration dependent manner.

Confocal microscopy (CSLM) allows visualisation of a section through a cell showing concentration of fluorophore at the cell edges indicating membrane receptor binding. Visualisation is of a particular plane of focus such that a "slice" through an individual cell may be observed, as known in the art. Different coloured channels may be selected to visualise different fluorophore types.

FCS is a non-invasive technique which measures fluctuations in fluorescence intensity in a confocal volume of $<10^{-15}$l. Statistical analysis of these fluctuations gives information about the speed of diffusion (i.e. mass) and concentration of the fluorescent molecules present. Thus free ligand (fast

diffusing) and bound ligand (slow diffusing) can be quantified simultaneously on a single cell.

FCS (fluorescence correlation spectroscopy) is a technique first described almost 30 years ago and developed out of the idea of correlating fluctuations in fluorescence emission of particles to parameters such as particle mass and concentration for the study of molecular interactions in solution. FCS essentially monitors spontaneous fluorescence intensity fluctuations of fluorescently tagged molecules in a microscopic detection volume (10^{-15}l) through analysis by a tightly focused laser beam.

These fluctuations provide information on the rate of diffusion or diffusion time of a particle which is directly dependent on the mass of the given molecule. When small and therefore rapidly diffusing molecules pass through the path of the laser they produce rapidly fluctuating fluorescence intensity patterns, whereas when larger molecules pass through the beam they produce bursts of fluorescence that are more sustained. Consequently the increase in the mass of a biomolecule, eg as a result of ligand binding, is detected as an increase in the diffusion time of the resultant biomolecule.

In a further aspect of the invention there is provided the use of a fluorescent ligand as hereinbefore defined in a method for binding a cell receptor as hereinbefore defined.

The invention is now illustrated in non-limiting manner with reference to the following figures and examples and accompanying synthesis schemes.

In the Figures:

Figure 1 shows images taken from confocal microscopy imaging of a) fluorescence derived from ligand binding of a fluorescent ligand of the invention to CHO cells observed at the red channel, b) fluorescence derived from green fluorescent protein expressed by CHO cells indicating receptor locations observed via the green channel and c) overlaid images from a) and b) showing overlap of fluorescence and therefore confirming ligand binding is specific to receptors.

In the Schemes:

Scheme 1 shows synthesis routes for the synthesis of an adenosine receptor antagonist Lig – L - Fl_L

Schemes 2 and 3 show synthesis routes for the synthesis of two adenosine receptor agonists Lig – L - Fl_L including the synthesis of ligand precursor Lig – L – Z_L from linker precursor Z_L'-L-Z_L

Scheme 4 shows synthesis routes for the synthesis of two beta adrenoceptor agonists Lig – L - Fl_L including the synthesis of ligand precursor Lig – L – Z_L from linker precursor Z_L'-L-Z_L

Examples

The following compounds are synthesised or modelled and binding affinity studied:

Example A1 / B1 / C1 Adenosine receptors antagonists:

XAC – BODIPY 630/650 (1)

Example A2 / B2 Adenosine receptor agonists:

Adenosine-BODIPY 630/650 (2)

NECA-BODIPY 630/650 (3) (ABEA – BODIPY 630)

APEA-BODIPY 630/650 (3a)

ABIPEA - BODIPY 630/650 (3b)

Example A3 / B3 Beta-adrenoreceptor agonists

Salmeterol – BODIPY 630/650 (4)

Clenbuterol – BODIPY 630/650 (9)

Example A4 / B4 Beta-adrenoreceptor antagonists

CGP12177-BODIPY 630/650 (5)

Propranolol-BODIPY 630/650 (6)

ICI118551-BODIPY 630/650 (7)

Alprenolol – BODIPY 630/650(8)

Example A5 / B5 Inhibitors of cyclic nucleotide phosphodiesterases

XAC – BODIPY 630/650 (1)

Materials and Methods

The ^{1}H NMR spectra were acquired on a Bruker AM 250 (250 MHz) spectrometer, in DMSO-d_6. Chemical shifts (δ) are recorded in ppm with reference to the residual solvent signal. Coupling constants (*J*) are recorded in hertz, and signal multiplicities are described by s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), br (broad). Where given, assignments are made based on homonuclear correlation spectroscopy (COSY-45) and, where available, are in full agreement with literature values (Jacobsen KA *et al.*, *J. Med. Chem.* (1985), **28**, 1341-6).

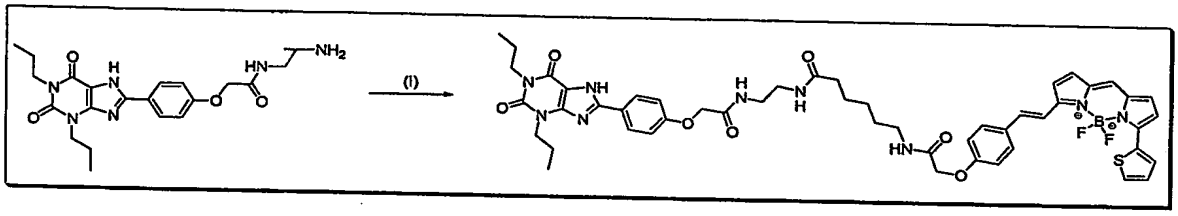
(Analytical RP-HPLC was performed on a Waters Millenium LC system with 996 PDA eluent detection, using a Vydac C^8 column (150mm x 4.6 mm) at a

flow rate of 1.0 mL.min^{-1}. The mobile phases used were: Solvent A, water, (degassed by helium bubble); Solvent B, acetonitrile, (degassed by sonication)).

A. SYNTHESIS

Example A1 –Synthesis of adenosine based fluorescent A_1 receptor antagonists

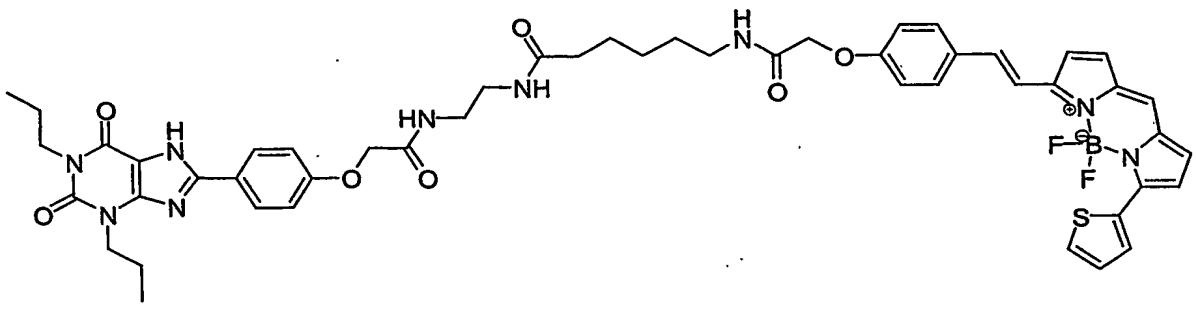
1. XAC-BY630 (1)



Scheme 1

Reagents and conditions: (i) BODIPY 630/650-SE, DMF 2 h, RT, (72%).

XAC-BODIPY 630/650 (established working/abstract name of XAC-BY630) was synthesised by reacting the primary alkyl amine group of XAC with BODIPY®-630/650-X-succinimidyl ester (Molecular Probes). XAC and BY630 were stirred in N,N-dimethylformamide for 2h at room temperature and the product purified by HPLC. XAC and analogues were synthesised by the method of Jacobsen *et al* above.



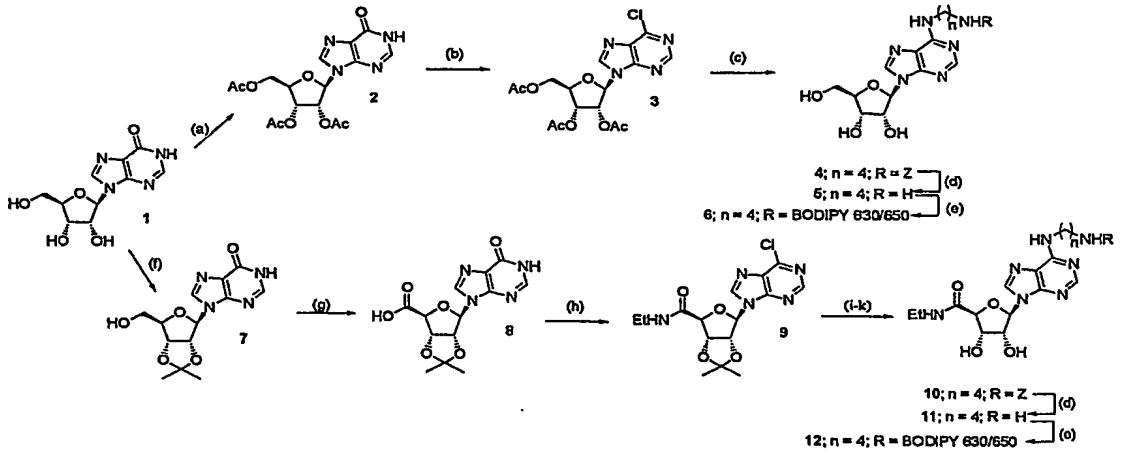
TOF ES+ found 974.3998 ($C_{50}H_{55}BF_2N_9O_7S$ requires 974.4006)

R_t 12.5 min (35-100% *v/v* B, 30 min)

0.87, 0.90 (6H, overlapping t, *J* 9.3, N^1-, N^3-$CH_2CH_2CH_3$), 1.14-1.25 (2H, m, $C^{24}H_2$), 1.36-1.62 (6H, m, $C^{23}H_2$, $C^{25}H_2$, $N^{1/3}$-$CH_2CH_2CH_3$), 1.68-1.78 (2H, m, $N^{1/3}$-$CH_2CH_2CH_3$), 2.04 (2H, t, *J* 7.3, $C^{22}H_2$), 3.04-3.19 (6H, m, $C^{18}H_2$, $C^{19}H_2$, $C^{26}H_2$), 3.86 (2H, t, *J* 7.4, $N^{1/3}$-$CH_2CH_2CH_3$), 4.01 (2H, t, *J* 7.1, $N^{1/3}$-$CH_2CH_2CH_3$), 4.52, 4.53 (4H, 2 x s, $C^{15}H_2$, $C^{29}H_2$), 6.95 (1H, d, *J* 4.2), 7.05-7.10 (4H, m), 7.27-7.30 (3H, m), 7.35-7.40 (2H, m), 7.41 (1H, br s), 7.54-7.65 (3H, m), 7.70 (1H, s), 7.77 (1H, s), 7.80-7.92 (2H, s), 8.01-8.23 (4H, m) (2 x $C^{11}H$, 2 x $C^{12}H$, 2 x $C^{32}H$, 2 x $C^{33}H$, $C^{35}H$, $C^{36}H$, $C^{38}H$, $C^{39}H$, $C^{41}H$, $C^{43}H$, $C^{44}H$, $C^{47}H$, $C^{48}H$, $C^{49}H$, N^9H, $N^{17}H$, $N^{20}H$, $N^{27}H$)

Example 2 - synthesis of adenosine based fluorescent agonists at the human A_1-adenosine receptor (A_1-AR) receptor based on 5'-*N*-ethylcarboxamidoadenosine (NECA) with maintained functional activity

Compounds 2, 3, 3a and 3b were synthesised by reaction of adenosine or NECA with chlorinating agent with use of protecting groups for the adenosine OH groups, introducing protected linker and removing protecting groups prior to linking with fluorescent agent via the linking group.

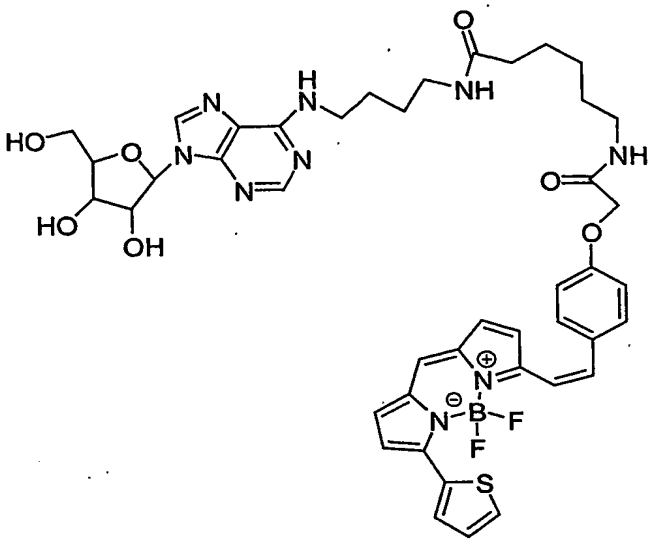


Scheme 2

Reagents and conditions: (a) Ac_2O, pyridine, 40°C, 1 h, 97%. (b) $POCl_3$, N,N-dimethylaniline, reflux, 5 min, 85%. (c) (i) $H_2N(CH_2)_4NHR$, DIEA, EtOH, reflux, 18 h, (ii) sat. NH_3/MeOH, 0°C, 2 h. 66%. (d) H_2, Pd/C, MeOH:H_2O:AcOH (7:2:1), r.t., 2 h, 80% (e) BODIPY 630/650-SE, DMF, r.t., 3 h, 63%

1. Adenosine-C^4- BODIPY 630/650 (ABA-BY630) (2)

ABA-BY630 was synthesised using the method and reagents and conditions described in Scheme 2 a-e in which R is $COCH_2Ph$.



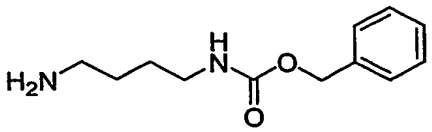
ES+ found 885.4 ($C_{43}H_{48}BF_2N_9O_7S$ requires 885.4)

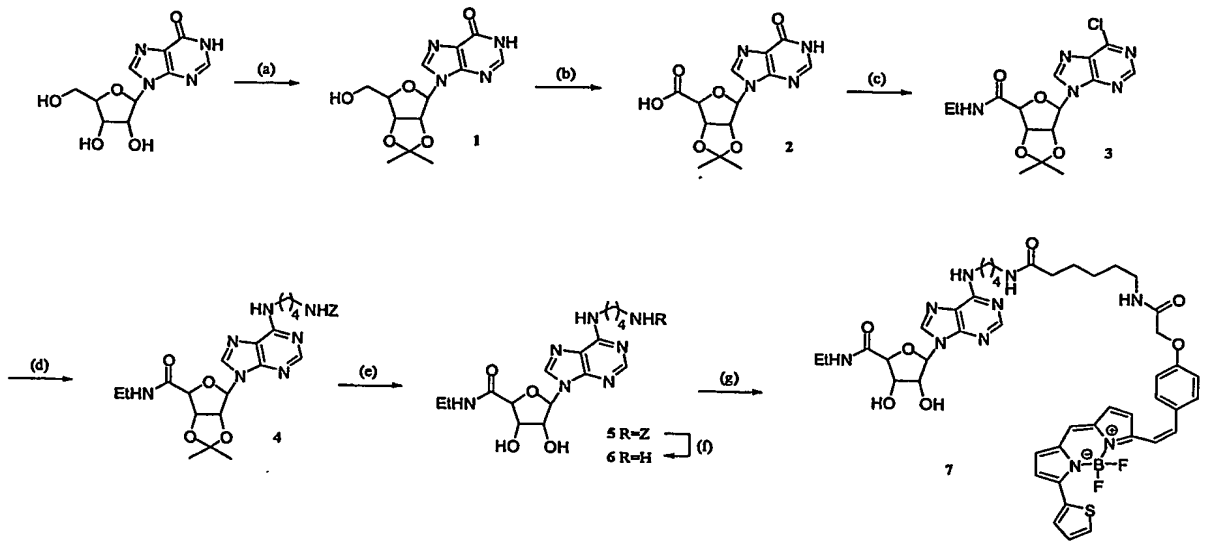
R_t 22.5 min (5-100% *v/v* B, 30 min)

2. NECA – C^4 - BODIPY 630/650 (ABEA-BY630) (3).

N^6-aminobutyl-5'-deoxy-5'-oxo-5'-ethylaminoadenosine (ABEA) was synthesised from commercially available reagents in 6 steps. The primary amine group of ABEA was acylated with the fluorophore BODIPY®630/650-X-succinimydyl ester (BY-630, Molecular Probes) to afford BY630-ABEA, which was purified by RP-HPLC (Scheme 1).

The synthesis is shown in Scheme 3, with use of linker precursor of formula $H_2N(CH_2)_5NHCOOCH_2Ph$:





Scheme 3

Reagents and Conditions: (a) 2,2-Dimethoxypropane, TsOH, acetone, r.t., 18 h. (b) TEMPO, BAIB, MeCN:H_2O (1:1), r.t., 4 h. (c) (i) $SOCl_2$, DMF, $CHCl_3$, reflux, 6 h. (ii) $EtNH_2$, $CHCl_3$, 5°C, 30 min. (d) $H_2N(CH_2)_4NHZ$, DIEA, EtOH, reflux, 18 h. (e) 0.1 M $HCl_{(aq)}$, 50°C, 4 h. (f) H_2, Pd/C, MeOH:H_2O:AcOH (9:0.9:0.1), r.t., 3 h. (g) BODIPY 630/650-X-SE, DMF, r.t., 4 h.

Synthesis of Lig-L-Z_L

2',3'-Isopropylideneinosine 1: Inosine (5.36 g, 0.02 mol) and tosic acid monohydrate (3.80 g, 0.02 mol) were suspended in a mixture of 2,2-dimethoxypropane (50 cm^3) and acetone (200 cm^3) and stirred for 18 h. Sodium hydrogen carbonate (2.52 g, 0.02 mol) and water (40 cm^3) were added and the suspension stirred for 15 min. The suspension was evaporated to constant volume and the crude product recrystallised from the residual water, yielding the acetonide **1** (3.71 g, 60%) as white needles; mp 266-268 °C (from H_2O) (lit., 266 °C); $[\alpha]^{22}_D$ -67.1 (c 0.59 in MeOH) (lit., $[\alpha]^{20}_D$ –66.9 (c 0.8 in

MeOH)); δ_H(250 MHz; DMSO-d_6) 1.31 (3 H, s, CH_3), 1.53 (3 H, s, CH_3), 3.53 (2 H, m, $C^{5'}H_2$), 4.22 (1 H, m, $C^{4'}H$), 4.93 (1 H, dd, *J* 6.1 and 2.5, $C^{3'}H$), 5.26 (1 H, dd, *J* 6.1 and 2.9, $C^{2'}H$), 6.10 (1 H, d, *J* 2.9, $C^{1'}H$), 8.10 (1 H, s, adenine CH), 8.31 (1 H, s, adenine CH); δ_C(69.2 MHz; DMSO-d_6) 25.2, 27.0 (2 x acetonide), 61.4 ($C^{5'}$), 81.3 ($C^{4'}$), 83.8 ($C^{3'}$), 86.6 ($C^{2'}$), 89.6 ($C^{1'}$), 113.1 (4°), 124.4 (4°), 138.7 (CH), 146.1 (CH), 147.8 (4°), 156.5 (4°); *m/z* (ES+) 309 (MH^+), 137 (M-ribose).

2',3'-Isopropylidene-5'-oxoinosine 2: Acetonide **1** (3.08 g, 10 mmol), TEMPO (313 mg, 2 mmol) and iodosobenzene diacetate (7.09 g, 22 mmol) were dissolved in MeCN: H_2O (1:1, 50 cm^3) and stirred, with the exclusion of light, for 4 h. The solvents were carefully evaporated from the resultant suspension and the reaction residue sequentially triturated with acetone and diethyl ether to yield the acid **2** (2.67 g, 83%) as a white powder; mp 224-229 °C (from diethyl ether) (lit., 274-276 °C); (found: C, 48.55; H, 4.3; N, 17.0. $C_{13}H_{14}N_4O_6$ requires C, 48.45; H, 4.4; N, 17.4%); δ_H(250 MHz; DMSO-d_6) 1.33 (3 H, s, CH_3), 1.51 (3 H, s, CH_3), 4.68 (1 H, d, *J* 1.6, $C^{4'}H$), 5.36-5.44 (2 H, m, $C^{2'}H$ and $C^{3'}H$), 6.30 (1 H, s, $C^{1'}H$), 8.02 (1 H, s, adenine CH), 8.27 (1 H, s, adenine CH), 12.42 (1H, br s, NH; δ_C(69.2 MHz; DMSO-d_6) 25.1, 26.7 (2 x acetonide), 83.9, 85.8, 90.0 (4 x CH), 112.9 (4°), 124.4 (4°), 140.0 (CH), 145.8 (CH), 148.2 (4°), 156.8 (4°), 171.8 (C=O); *m/z* (ES+) 323 (MH+), 137 (M-ribose).

6-Chloro-6-deoxy-5'-ethylamino-2',3'-isopropylidene-5'-oxo-5'-deoxyinosine 3: (N.B. Rigorously dry reaction conditions and under an inert atmosphere) acid **2** (967 mg, 3 mmol), was suspended in $CHCl_3$ (15 cm^3) to which was added *N,N*-DMF (581 μL, 7.5 mmol) and $SOCl_2$ (1.09 cm^3, 15 mmol). The suspension was placed in a hot oil-bath and maintained at reflux for 6 h. The resultant solution was evaporated and the yellow oil dissolved in

THF (20 cm^3) at 5 °C. Ethylamine (2.0 M solution in THF, 3.75 cm^3, 7.5 mmol) was added drop wise, stirred at 5 °C for 15 min and allowed to warm to room temperature. The solvent was evaporated, the residue dissolved in DCM (25 cm^3) and washed with water (2 x 20 cm^3) and saturated brine solution (2 x 20 cm^3). The organic fraction was dried and evaporated to leave a yellow oil that was purified by column chromatography on silica (5 % MeOH-DCM) to give the title compound **3** (525 mg, 48%) as a yellow syrup; $[\alpha]^{19}_D$ –12.9 (c 0.50 in $CHCl_3$); δ_H(250 MHz; $CDCl_3$; Me_4Si) 0.78 (3 H, t, *J* 7.3, CH_2CH_3), 1.41 (3 H, s, CH_3), 1.64 (3 H, s, CH_3), 2.90-3.11 (2 H, m, CH_2CH_3), 4.74 (1 H, d, *J* 1.9, $C^{4'}H$), 5.46 (1 H, dd, *J* 6.2 and 2.3, $C^{2'}H$), 5.54 (1 H, dd, *J* 6.2 and 1.9, $C^{3'}H$), 6.24 (1 H, d, *J* 2.3, $C^{1'}H$), 6.28 (1 H, br s, NH), 8.35 (1 H, s, adenine CH), 8.68 (1 H, s, adenine CH); δ_C(69.2 MHz; $CDCl_3$; Me_4Si) 14.2 (CH_2CH_3), 25.0, 26.9 (2 x acetonide), 33.9 (CH_2CH_3), 82.9, 83.4, 86.7, 92.0 (4 x CH), 114.6 (4°), 132.3 (4°), 144.8 (CH), 150.9 (4°), 151.9 (4°), 152.2 (CH), 168.1 (C=O); *m/z* (ES-) 366 ($(M-H)^-$), 153 (M-ribose).

N^6-(4-Benzyloxycarbonylaminobutyl)-5'-ethylamino-2',3'-isopropylidene-5'-oxo-5'-deoxyadenosine 4: Chloride **3** (337 mg, 0.92 mmol) was dissolved in EtOH (10 cm^3) to which was added *N*-benzyloxycarbonylbutan-1,4-diamine (305 mg, 1.37 mmol) and DIEA (159 μL, 0.92 mmol). The solution was placed in a hot oil-bath and maintained at reflux for 18 h. The resultant solution was evaporated and the yellow oil purified by column chromatography on silica (2.5 % MeOH-DCM) to give the title compound **4** (445 mg, 88%) as a pale yellow gum; δ_H(250 MHz; $CDCl_3$; Me_4Si) 0.99 (3 H, t, *J* 7.1, CH_2CH_3), 1.43 (3 H, s, CH_3), 1.55-1.71 (7 H, m, CH_3 and 2 x CH_2), 3.20-3.35 (2 H, m, CH_2), 3.55-4.01 (4 H, m, CH_2CH_3 and CH_2), 4.81 (1 H, s, CH), 5.10 (3 H, m, benzyl CH_2 and CH), 5.51 (1 H, d, *J* 5.9, CH), 5.71 (1 H, d, *J* 5.9, CH), 6.10 (1 H, br s, NH), 6.16 (1 H, br s, NH), 7.30-7.36 (5 H, m, aromatics), 7.86 (1 H, s, adenine CH), 8.22 (1 H, s, adenine CH); δ_C(69.2

MHz; $CDCl_3$; Me_4Si) 13.7 (CH_2CH_3), 25.1, 26.6 (2 x acetonide), 26.8, 27.0, 40.0, 40.4 (4 x CH_2), 61.5 (CH_2CH_3), 66.6 (benzyl CH_2), 84.1, 84.7, 87.0, 91.6 (4 x CH), 113.7 (4°), 128.1 (C), 128.5 (CH), 136.7 (4°), 139.9 (CH), 152.8 (CH), 154.9 (4°), 156.5 (CH), 169.4 (C=O); *m/z* (ES+) 554 (MH+), 341 (M-ribose).

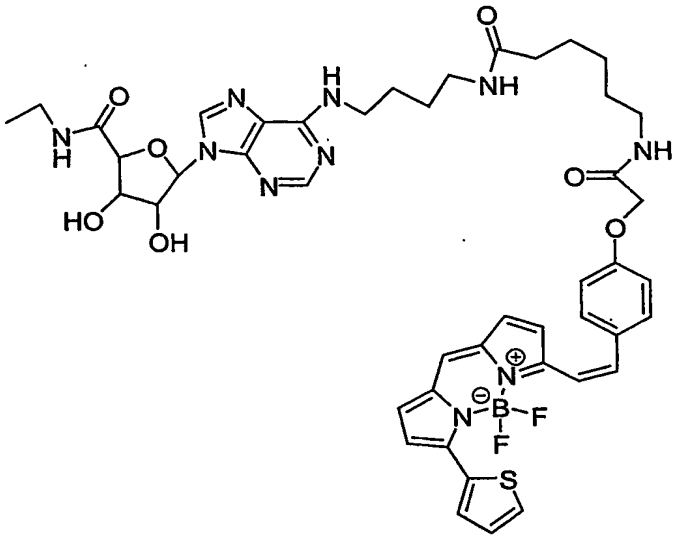
N^6-(4-Benzyloxycarbonylaminobutyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine 5: Adenosine derivative **4** (261 mg, 0.47 mmol) was dissolved in 1 M $HCl_{(aq)}$: 1,4-dioxane (1:1, 4 cm^3), placed in a 50 °C oil-bath and stirred for 4 h. The resultant solution was adjusted to ~pH 8 (satd. $NaHCO_{3(aq)}$), saturated with NaCl and extracted with EtOAc (3 x 5 cm^3). The combined organic fractions were dried and evaporated and the crude product purified by preparative layer chromatography (10 % MeOH-DCM) to give the title compound **5** (160 mg, 66%) as a colourless oil; δ_H(250 MHz; DMSO-d_6) 1.08 (3 H, t, *J* 7.2, CH_2CH_3), 1.45-1.62 (4 H, m, C^2H_2 and C^3H_2), 2.98-3.06 (2 H, m, C^1H_2), 3.17-3.26 (2 H, m, CH_2CH_3), 3.37-3.53 (2 H, m, C^4H_2), 4.12-4.16 (1 H, m, $C^{3'}H$), 4.31 (1 H, d, *J* 1.1, $C^{4'}H$), 4.58-4.65 (1 H, m, $C^{2'}H$), 4.99 (2 H, s, benzyl CH_2), 5.56 (1 H, d, *J* 6.5, $C^{2'}$-OH), 5.76 (1 H, d, *J* 4.2, $C^{3'}OH$), 5.96 (1 H, d, *J* 7.6, $C^{1'}H$), 7.25-7.34 (6 H, m, aromatics and NH), 8.01 (1 H, br s, carbamate NH), 8.27 (1 H, s, adenine CH), 8.39 (1 H, s, adenine CH), 8.94 (1 H, t, *J* 5.6, amide NH); δ_C(69.2 MHz; DMSO-d_6) 14.9 (CH_2CH_3), 26.6, 27.1, 33.4, 39.5, 40.3 (5 x CH_2), 65.3 (benzyl CH_2), 72.2, 73.3, 84.9, 88.0 (4 x CH), 120.2 (4°), 127.9 (CH), 128.5 (CH), 137.5 (CH), 140.6 (4°), 152.6 (CH), 154.9 (4°), 156.3 (4°), 169.3 (4°); *m/z* (ES+) 514 (MH+).

N^6-(4-Aminobutyl)-5'-ethylamino-5'-oxo-5'-deoxyadenosine (ABEA) 6: Adenosine derivative **5** (48 mg, 0.09 mmol) was dissolved in MeOH:H_2O:AcOH (9:0.9:0.1, 5 cm^3), to which was added 10 % Pd/C (10 mg). The flask was evacuated, filled with hydrogen (balloon) and stirred vigorously

for 3 h. The reaction mixture was filtered through celite and the celite washed with MeOH. The combined organic filtrates were evaporated and the resultant oil evaporated again from MeCN (2 x 15 cm^3) to give the title compound **6** (35 mg, quant.) as a colourless oil; δ_H(250 MHz; DMSO-d_6) 1.08 (3 H, t, *J* 7.2, CH_2CH_3), 1.46-1.88 (6 H, m, 2 x CH_2 and NH_2), 2.63 (2 H, t, *J* 6.8, CH_2), 3.16-3.29 (2 H, m, CH_2CH_3), 3.40-3.52 (2 H, m, CH_2), 4.10-4.15 (1 H, m, $C^{3'}H$), 4.30 (1 H, d, *J* 1.3, $C^{4'}H$), 4.53-4.62 (1 H, m, $C^{2'}H$), 5.96 (1 H, d, *J* 7.7, $C^{1'}H$), 8.05 (1 H, br s, NH), 8.27 (1 H, s, adenine CH), 8.39 (1 H, s, adenine CH), 8.95 (1 H, t, *J* 5.6, amide NH); *m/z* (ES+) 380 (MH+).

Synthesis of Lig-L-Fl_L

ABEA-BY630 (3): ABEA **6** (5.74 mg, 15.1 μmmol) was dissolved in *N,N*-DMF (1 cm^3) under an inert atmosphere and with the exclusion of light. A solution of Bodipy 630/650-X-succinimidyl ester (Molecular Probes) (5.0 mg, 7.55 μmmol, 1 cm^3 *N,N*-DMF) was added and the reaction stirred for 4 h. The solution was evaporated and the crude product purified by preparative layer chromatography (10 % MeOH-DCM) to give the title compound **7 (3)** (5.24 mg, 75%) as a purple powder; *m/z* (ES+) found 947.37 ($C_{45}H_{51}BF_2N_{10}O_7SNa$ requires 947.36).



ABEA-BY630

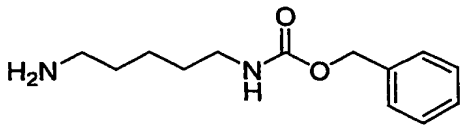
5

10

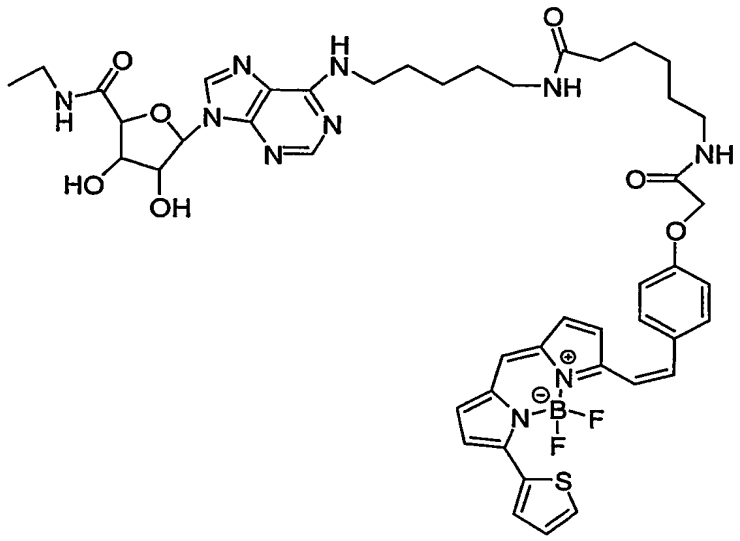
3. NECA - C^5 - BODIPY 630/650 (ABEA-BY630) (3a)

This compound was synthesised using the method of Scheme 3 as described for Compound (3), with use of linker precursor of formula

15 $H_2N(CH_2)_5NHCOOCH_2Ph$:



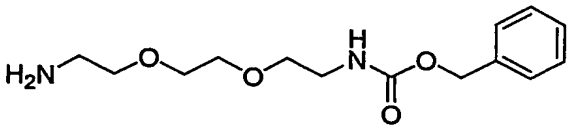
ABEA-BY630 was obtained having the formula:



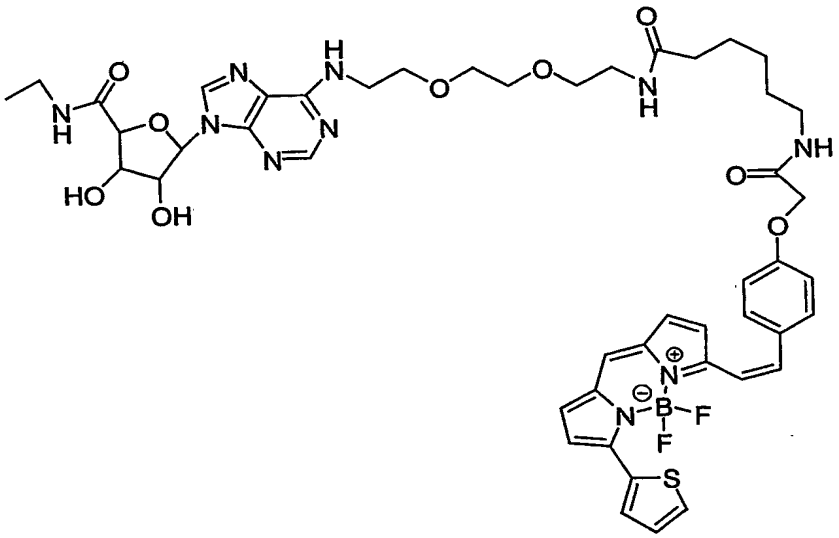
R_t 8.6 min (30-100% *v/v* B, 25 min)

4. NECA-PEG^8- BODIPY 630/650 (ABIPEA-BY630) (3b)

This compound was synthesised using the method of Scheme 3 as described for Compound (3), with use of linker precursor of formula $H_2N((CH_2)_2O)_2(CH_2)_2COOCH_2Ph$:



ABEA-BY630 was obtained having the formula:



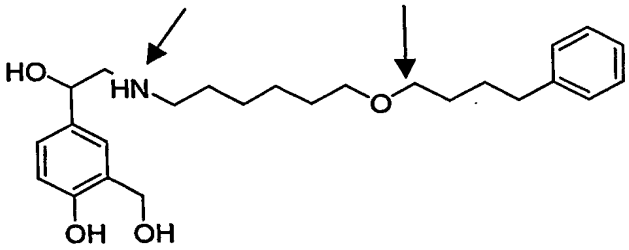
TOF ES+ found 985.3993 ($C_{47}H_{56}BF_2N_{10}O_9S$ requires 985.4013)

R_t 8.3 min (35-100% *v/v* B, 25 min)

Example A3 –Synthesis of β-Adrenoceptor agonists

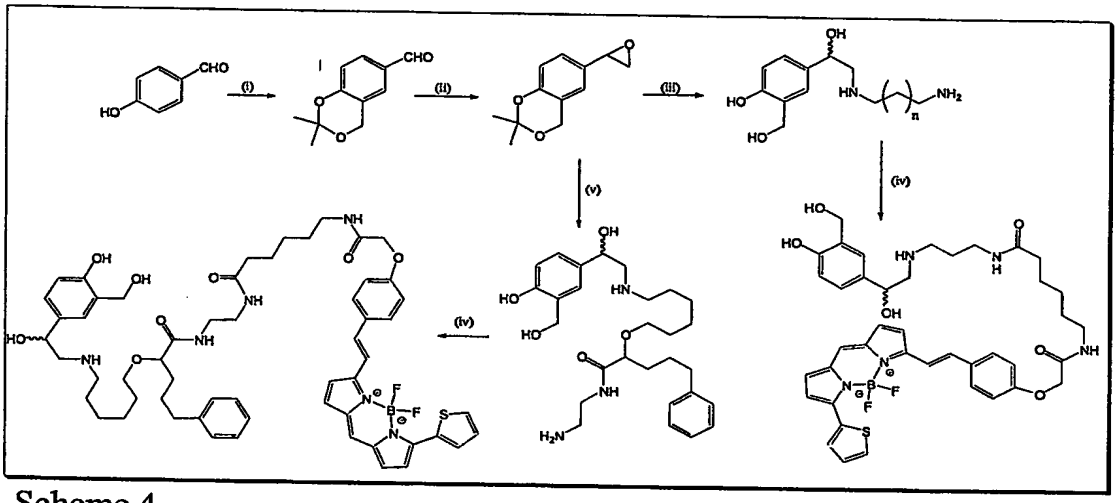
1. Salmeterol-BODIPY 630/650 (4) and Derivative-Salmeterol-BODIPY 630/650 (4a)

Salmeterol is linked to fluorophore via two different linking sites, in the folllowing syntheses



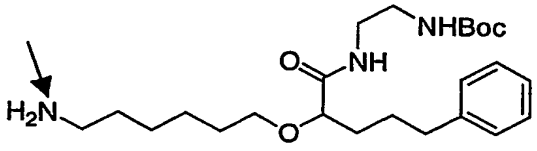
In a first approach, inserting a synthetic linker onto the salmeterol chain adjacent to the oxygen, we can couple the fluorophore and we don't lose any of the parent active ligand, we just add to it. This is quite complex so in a the

second approach we simply take the salmeterol head-group, and link this through the nitrogen to another linker and then to the fluorophore ie replacing the long alkyl chain of salmeterol with the linker and long alkyl chain of BODIPY. In this case, according to the invention, retention o binding, fluorescence and activity are uncertain and must therefore we verified and information provided with the fluorescent ligand, to provide a useful compound.

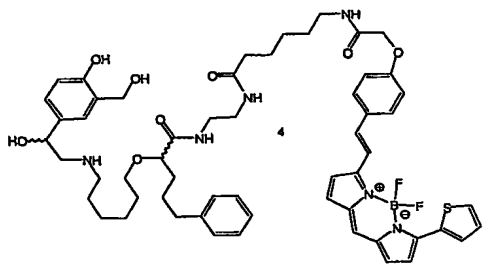


Scheme 4

Reagents and conditions: (i) (a) HCHO, $HCl_{(aq)}$, dioxane, 60°C (b) 2,2-Dimethoxypropane, TsOH. (ii) Me_3SI, NaH, THF. (iii) (a) $BocNH(CH_2)_nNH_2$, EtOH, (b) HCl, Et_2O. (iv) BODIPY 630/650-X-SE, DMF, RT. (v) (a) $Z_L'Y_L'$-L-Y_LP_L, EtOH, (b) HCl, Et_2O, $Z_L'Y_L'$-L-Y_LP_L is

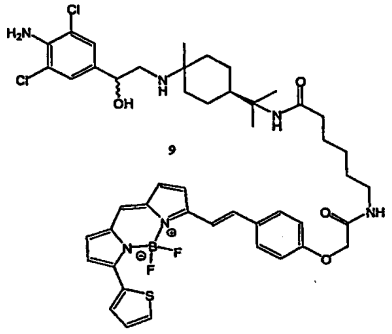


and results in compound 4a



All of the following molecules rely upon the synthesis of the same two linker moieties as shown in Scheme 4 and described above, (where the hydrocarbon chain length can be easily varied, or altered chemically to *e.g.* an ethylene glycol structure to improve solubility).

2. Clenbuterol-BODIPY 630/650 (9)

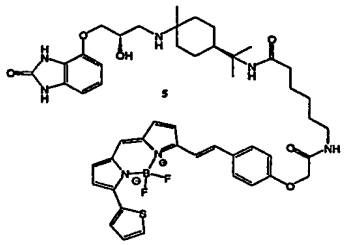


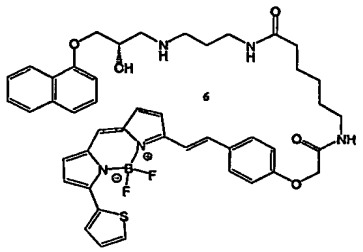
Example A4 –Synthesis of β-Adrenoceptor antagonists

All of the following molecules rely upon the synthesis of the same two linker moieties as shown in Scheme 4 and described in Example A3, (where the

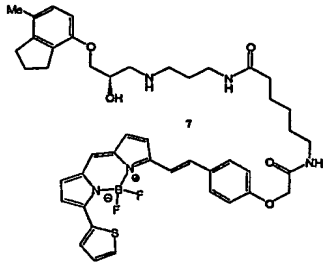
hydrocarbon chain length can be easily varied, or altered chemically to *e.g.* an ethylene glycol structure to improve solubility).

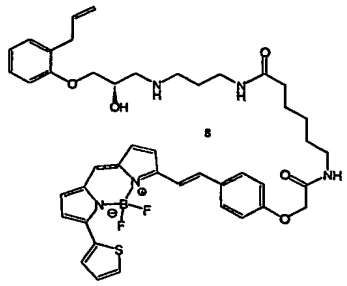
1. CGP 12177-BODIPY 630/650 (5) **2. Propranolol-BODIPY 630/650 (6)**





3. ICI118551-BODIPY 630/650(7) **4. Alprenolol-BODIPY 630/650(8)**





B. PHARMACOLOGY

Example B1 –Binding of adenosine based fluorescent A_1 receptor antagonists

1. XAC-BY630 (1)

The adenosine-A_1 receptor (A_1-AR) is a G-protein coupled receptor which is found in a variety of tissues including brain, heart, adipose tissue and muscle. By conjugating the A_1-AR antagonist xanthine amine cogener (XAC) to the fluorophore BODIPY®-630/650 (BY630), we have synthesised a fluorescent A_1-AR ligand, XAC-BY630, to allow visualisation of this receptor in living cells.

[^{3}H]DPCPX binding alongside cyclic AMP and inositol phosphate accumulation assays were performed on CHO-A1 cells expressing the human A1-receptor. Images were acquired using a Zeiss LSM510 confocal microscope using CHO-A1 cells grown to 50% confluency on 8-well Labtek™ plates in Dulbecco's
Modification of Eagle's Medium:Ham's F12 containing 5% foetal calf serum and 2mM glutamine. Cells were washed twice with HEPES-buffered saline prior to incubation at 22°C with compounds as indicated.

Spectroscopic analysis of XAC-BY630 and BY630 itself showed that their peak excitation (630, 632nm, respectively) and emission wavelengths (650, 653nm) were not substantially different. [^{3}H]DPCPX binding studies on CHO-A1 cell membranes showed that XAC-BY630 had a lower affinity for the A_1-AR than XAC (pK_i=7.79±0.13 and 6.82±0.11, XAC and XAC-BY630, respectively, mean±s.e.mean, n=4). XAC-BY630 also behaved as a competitive A_1-AR antagonist at both 5'-N-ethylcarboxamidoadenosine-mediated inhibition of cAMP production (apparent pK_B=6.98±0.15 vs. 8.06±0.24 for XAC, n=3) and stimulation of inositol phosphate production (apparent pK_B=6.26±0.20 vs. 7.46±0.08 for XAC, n=4). Confocal imaging showed that XAC-BY630 bound to membrane-localised A_1-ARs in a time- and concentration-dependent manner. Binding of XAC-BY630 (25-250nM)

was detected after 5 min, and was predominantly located at the membrane after a 30 min. incubation. Membrane binding of XAC-BY630 was receptor-specific, since a 30 min pre-incubation with DPCPX (10^{-8}-10^{-6}M) caused a concentration-dependent inhibition of membrane binding (30 min, 50nM).

These studies indicate that XAC-BY630 is a functional A_1-AR antagonist with moderate affinity which could be used to visualise the A_1-AR in primary tissue and cell lines.

Fluorescence Correlation Spectroscopy (FCS).

FCS is a non-invasive technique which measures fluctuations in fluorescence intensity in a confocal volume of $<10^{-15}$l. Statistical analysis of these fluctuations gives information about the speed of diffusion (i.e. mass) and concentration of the fluorescent molecules present. Thus free ligand (fast diffusing) and bound ligand (slow diffusing) can be quantified simultaneously on a single cell. We have used FCS to measure binding of the fluorescent ligand, xanthine amine cogener-BODIPY®630/650 (XAC-BY630) to the human adenosine A_1 receptor (A_1-AR).

CHO cells expressing either the human A_1-AR or an A_1-AR-Topaz fusion were cultured on glass-bottomed 8-well plates and prepared for live cell measurement. FCS measurements were made using a Zeiss Confocor 2, fitted with an Axiocam CCD camera for x-y positioning. Cells were incubated with ligands at 22°C for the times indicated and the confocal volume was positioned on the upper membrane. Data were collected for 2x30s, following a 15s pre-bleach and analysed using a multi-parameter equation using Zeiss AIM software.

Initially, the diffusion characteristics of the A_1-AR-Topaz fusion protein (A_1-AR-Tpz) were determined in CHO-A1Tpz cells. Autocorrelation analysis showed the diffusion time (τ_D) for the A_1-AR was 15.0±0.9ms (mean±s.e.mean, n=84). A second component (τ_D=118±14μs) was also seen, probably caused by an optical event within the fluorophore ("blinking"). FCS analysis of XAC-BY630 in buffer showed a single component diffusion (τ_D=60±2μs, n=10). On the upper membrane of CHO-A1 cells incubated with XAC-BY630 (1-40nM, 10-60 min, n=71), two further slow-diffusing species were detected in addition to free ligand. The first component had a similar diffusion time (τ_{D1}=17.4±1.1ms; 69/71 cells) to that seen for A_1-AR-Tpz, suggesting that it is receptor-bound ligand. The second was a very slow diffusing component (τ_{D2}=345±41ms, 61/71 cells). Following preincubation with 8-cyclopentyl-1,3-dipropyl xanthine (DPCPX) (1μM, 30 min), t_{D2} was present in 30/31 cells, suggesting this component is non-specific binding. However, the t_{D1} component was present in only 17/31 cells. In addition, in cells exposed to 15nM XAC-BY630 for 30 min the amount of τ_{D1} component was reduced from 51.8±14.9 to 13.6±5.4 receptors/μm^2 by DPCPX (n=8 and 4, respectively, Student's t-test, $P<0.05$), further suggesting this component is A_1-AR bound ligand.

We have used FCS to quantify binding to the A_1-AR and measure receptor diffusion in single live cells. Further development allows quantitative receptor-ligand binding of the endogenous A_1-AR in acutely dispersed cells.

These studies indicate that XAC-BY630 is a functional A_1-AR antagonist with moderate affinity which could be used to visualise and measure binding to the A_1-AR, in primary tissue and cell lines.

Example B2–Binding of NECA based fluorescent A_1-receptor agonists

2. BY630-ABEA (3)

Functional studies were performed in CHO-K1 cells expressing both the human A_1-AR and a c-fos-pGL3 reporter vector (CHO-A1fos cells). Cells were incubated for 24h in serum-free DMEM/F-12 media, then stimulated with agonist for 5h, in some cases following 30 min incubation with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Luciferase expression was quantified using a Luclite® kit according to manufacturer's instructions. Live cell confocal imaging was carried out on CHO-A1 cells or CHO cells expressing the A_1-AR tagged on the C-terminus with a green fluorescent protein (CHO-A1Tpz).

In CHO-A1fos cells, both BY630-ABEA and the A_1-AR agonist N^6-cyclopentyl adenosine (CPA) stimulated luciferase expression in a dose-dependent manner (pEC_{50}'s of 7.01±0.04 (n=6) and 6.76±0.18 (n=5) for CPA and BY630-ABEA, respectively, mean±s.e.mean). Stimulation was mediated by the A_1-AR receptor, since the concentration response curves were shifted to the right in a competitive manner by 10nM DPCPX, yielding pK_d values of 8.72±0.03 and 9.05±0.10 vs. CPA and BY630-ABEA, respectively (n=3). A higher dose of DPCPX (100nM), gave a pK_d of 8.62±0.02 for CPA stimulation, but completely blocked the response to BY630-ABEA (n=3). For receptor visualisation, CHO-A_1 cells were incubated with 100nM BY630-ABEA for up to 60min. Binding of ligand to the membrane was detectable after 5 min, and was substantial after 30 min (n=3). Binding was to the A_1-AR, since it was substantially reduced by preincubation with DPCPX (1μM, 30 min). In addition, experiments in CHO-A1Tpz cells, showed co-localisation of ligand fluorescence at the membrane with that from the fluorescently tagged A_1-AR.

Results are shown in Figure 1 which shows images taken from confocal microscopy imaging of a) fluorescence derived from ligand binding of a fluorescent ligand of the invention to CHO cells observed at the red channel, b) fluorescence derived from green fluorescent protein expressed by CHO cells indicating receptor locations observed via the green channel and c) overlaid images from a) and b) showing overlap of fluorescence and therefore confirming ligand binding is specific to receptors.

In conclusion, we have succeeded in synthesising a novel fluorescent agonist ligand for the human A_1-AR. This ligand will be useful in monitoring the localisation of the endogenous A_1-AR receptor in both acutely dispersed cells and cell lines.

C. LIGANDS ASSOCIATED WITH PHARMACOLOGICAL DATA

Example C1 – Data sheets for kit / catalogue compound comprising adenosine based fluorescent A_1-receptor antagonists

1. XAC-BY630 (1)

Characterisation: Fluorescent adenosine A_1-receptor antagonist.

Synthesis and analysis: see A1 above.

Solubility x mg/ml (DMSO)

MW = xxx

Storage ....-20°C (dark)

Spectral Properties:

Excitation Max 638nm

Emission Max 655nm

Fluorescence Lifetime 4.2 ns

Emission quantum yield 0.33

Pharmacology:

CHO-cells expressing human adenosine A_1-receptor:

Inhibition of ^{3}H-DPCPX binding (membranes) $pK_B = - 6.82 + 0.11$

Inhibition of ^{3}H-DPCPX binding (whole cells) $pK_B = -6.9$

Antagonism of NECA-stimulated cAMP accumulation $pK_I = -6.98 + 0.15$

Antagonism of NECA-stimulated inositol phosphate accumulation $pK_I = -6.26 + 0.20$

Imaging:

Picture of XAC-BY630/650 binding to CHO-A1 cells and CHO-A1-GFP cells Also pictures showing displacement of binding by non-fluorescent antagonist DPCPX.

D. KIT WITH DIFFERENT FLUORESCENTLY TAGGED LIGANDS

A kit is assembled comprising 3 fluorescent ligands each ligand comprising ABIPEA fluorescently tagged with a fluorophore providing different fluorescence characteristics selected from BODIPY 630/650 SE, BODIPY 630/650 methyl bromide, BODIPY FL ethylene diamine etc.

Fluorescently tagged ligands are obtained by the process of the invention as hereinbefore defined.

The kit includes data sheets (C. above) for each ligand.

An alternative kit is assembled comprising 9 fluorescent ligands 3 of which comprise ABIPEA, a further 3 comprising a second ligand and a further 3 comprising a third ligand, each group of 3 ligands fluorescently tagged with a fluorophore providing different fluorescence characteristics selected from BODIPY 630/650 SE, BODIPY 630/650 methyl bromide, BODIPY FL ethylene diamine etc.

Fluorescently tagged ligands are obtained by the process of the invention as hereinbefore defined.

The kit includes data sheets (C. above) for each ligand.

An alternative kit is assembled comprising 3 tagged ligands each ligand comprising ABIPEA tagged with a selection of tags as known in the art, including one tagged with a fluorophore.

The kit includes data sheets (C. above) for each ligand.

The kits are useful for conducting binding studies as known in the art for a desired fluorescent ligand having the desired fluorophore or for a selection of fluorescent ligands or for a selection of ligands one of which comprises a desired fluorophore.

CLAIMS

1. Kit comprising a plurality of tagged non-peptide ligands of formula Lig-L-Tag

in each of which a precursor ligand is linked to one of a plurality of different tags or each of a plurality of precursor ligands are linked to a plurality of different tags

Wherein Lig- comprises a biogenic amine GPCR receptor ligand selected from a) an adenosine receptor antagonist b) an adenosine receptor agonist c) a beta-adrenoceptor agonist and d) a beta-adrenoceptor antagonist; or Lig- comprises e) an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases; or a derivative or analogue thereof;

-L- is a single bond or is any linking moiety which may be monomeric, oligomeric having oligomeric repeat of 2 to 30 or polymeric having polymeric repeat in excess of 30 up to 300;

and wherein each -Tag is any known or novel tagging substrate.

2. Kit as claimed in Claim 1 wherein one or more or each -Tag is an entity -Fl and comprises any known or novel fluorophore, whereby the kit comprises a plurality of compounds of which one or more or all of which are of formula Lig-L-Fl.

3. Kit as claimed in any of Claims 1 and 2 which comprises one or more families of tagged non-peptide ligands in which one or more precursor ligand is linked to a number of different tags or comprises a library of non-peptide ligands in which a plurality of precursor ligands are each linked to one or a plurality of different tags.

4. Kit as claimed in any of Claims 1 to 3 wherein a precursor ligand is linked to each of a plurality of fluorophores and/or tags providing a family of differently fluorescently tagged ligands; and/or providing a family of differently tagged ligands including at least one fluorescently tagged ligand.

5. Kit as claimed in any of Claims 1 to 4 comprising from 2 to 250 tagged ligands, preferably comprising from 1 to 10 families comprising 2 to 25 tagged ligands each family comprising a ligand of a common ligand type and from 2 to 25 different tag types at least one of which is a fluorescent tag, more preferably each of which is a different fluorescent tag; or the kit comprises a library of from 5 to 250 fluorescently tagged ligands of different ligand type and different fluorophore type.

6. Kit as claimed in any of Claims 1 to 5 including information for each tagged ligand Lig-L-Tag comprised in the kit, relating to the pharmacology for binding to a cell receptor of the biogenic amine class of GPCR receptors or to inhibition of intracellular enzymes such as cyclic nucleotide phosphodiesterases.

7. Kit as claimed in any of Claims 1 to 6 wherein Lig- is selected from

a) xanthine like structures including XAC, theophyline, caffeine, theobromine, dyphilline, enprofylline and the like; or fused biaryl structures including papaverine, dihydroquinilones such as cilostamide, dipyridamole, vinpocetine and the like; and analogues thereof;

b) adenosine like structures including ADAC, ABEA, NECA and analogues thereof;

c) ethanolamine structures including salmeterol, salbutamol, terbutaline, quinprenaline, labetalol, sotalol, bambuterol, fenoterol, reprotolol, tulobuterol, clenbuterol and analogues thereof;

d) oxypropanolamine structures including CGP12177, propranolol, practolol, acebutalol, betaxolol, ICI 118551, alprenolol, celiprolol (celectol), metoprolol (betaloc), CGP20712A, atenolol, bisoprolol, misaprolol, carvedilol, bucindolol, esmolol, nadolol, nebivolol, oxprenolol, xamoterol, pindolol, timolol and analogues thereof;

e) xanthine like structures including XAC, theophylline, caffeine, theobromine, dyphilline, enprofylline, sildenafil, EHNA (erythro-9-(2-hydroxyl-3-nonyl)adenine), zaprinast and the like; or spiro bicyclic structures including bypyridines such as amrinone, imidazolines such as CI930, dihydropyridazinones such as indolan, rolipram, SB207499, and the like; or fused biaryl structures including papaverine, dihydroquinilones such as cilostamide, dipyridamole, vinpocetine and the like and analogues thereof.

8. Kit as claimed in any of Claims 1 to 7 wherein – L- is selected from a saturated or unsaturated single or double bond, -O-, -S-, amino, COO-, hydrazine; and saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, and comprising functionality derived from a reactive group or site for linking to fluorophore and/or to ligand selected from a saturated or unsaturated single or double bond, -O-, -S-, amino, hydrazine, carbonyl and the like.

9. Kit as claimed in any of Claims 1 to 8 wherein – L- comprises a mono or difunctional linear or cyclic substituted or unsubstituted alkyl of formula – L.I-

-L.I- $-Y_L'\left[(CR_L)q_L\,A\right]q_L''\,(CR_L)q_L'\,Y_L-$

wherein each of -Y_L'- and -Y_L- is independently selected from a single bond, methylene, alkyne, alkene, NH, NR, O, NRCO, S, CO, NCO, CHHal, P and the like wherein Hal is any halogen selected from chlorine, iodine, fluorine;

-A- is a property conferring group selected from –O-, -C(=O)- cycloalkyl, heterocyclic, alkenyl, aryl, heteroaryl as hereinbefore defined and combinations thereof and the like;

q_L and q_L' are independently zero or are selected from a whole number integer from 1 to 10, preferably the sum of q_L and q_L' is zero, 1 or greater than or equal to 2 and less than or equal to 12; and

q_L'' is 0 or 1 to 30, or indicates a polymeric repeat unit of 30 up to 300

and each R_L is selected independently from H, C_{1-3} alkyl, C_{1-5} alkoxy and the like.

10. Kit as claimed in Claim 9 wherein – L- is of formula -L.II-

-L.II- $-Y_L'[(CH_2)_2O]q_L''(CH_2)_2Y_L-$

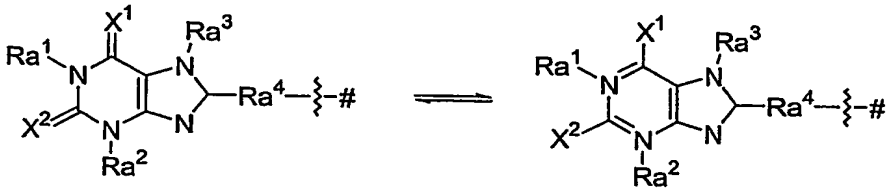
wherein Y_L', Y_L and q_L'' are as hereinbefore defined in Claim 9.

11. Kit as claimed in any of Claims 1 to 10 wherein Lig-L- is selected from formulae Lig.a-L.a- to Lig.e- L.e-:

Wherein:

Lig.a- is of the formula, in either of the following forms given:

Lig.a [1]-



Wherein

X^1 and X^2 are each independently selected from H, =O, OR.a, NR.a, NHR.a;

X^1 and X^2 are each preferably =O;

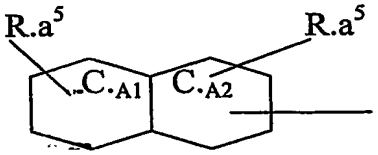
R.a, $R.a^1$, $R.a^2$ and $R.a^3$ are each independently selected from H or C_{1-4} linear or branched alkyl, preferably H, methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl or isobutyl optionally mono or multi hydroxy or halo substituted, such as CH_2OH, CH_2F or $CH_2CHOHCH_2OH$;

$R.a^4$ is selected from a heteroatom O, S or substituted or unsubstituted amine or saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano;

preferably $R.a^4$ is selected from optionally substituted aryl, cycloalkyl, alkyl, ketone, (di)amine, (di)amide, more preferably optionally substituted alkoxy, cycloalkyl, amine, amide, carboxylic acid or optionally o-, m- or p- substituted phenyl wherein substituents include aryl, alkyl, cycloalkyl, heteroaryl or heteroalkyl, amine, amide, carboxyl, carbonyl etc, for example is cyclohexyl, cyclopentyl, ethoxy, $(CH_2)_2PhPh$, CH_2Ph, $CONH(CH_2)nCONH$, $CH_2CONH(CH_2)_2NH$, $CH_2PhNHCOCH_2$, $CH_2CH_2OCOCH_2$, succinimidyl ester, $NHCOCH_2$,

$CH_2(CH_3)NCOCH_2$, $H_2N(CH_2)_2NHCOCH_2$, $H_2N(CH_2)_8NHCOCH_2$, $H_2NNHCOCH_2$, $CH_2CONH(CH_2)_2NHCOCH_2$, $HOPhCH_2N(CH_2CH_3.HOAc)(CH_2)_2NHCOCH_2$, heterocyclic-$(CH_2)_4CONH(CH_2)_2NHCOCH_2$, heterocyclic-$NHCON$(heterocyclic)$COCH_2$;

or Lig.a- is of the formula Lig.a^2-

Lig.a^2 

wherein each of $C._{A1}$ and $C._{A2}$ is independently selected from aryl, heteroaryl, cycloalkyl and heterocyclic, more preferably from phenyl, or aryl containing 1 or 2 ring heteroatoms, or heterocyclic containing 1 ring heteroatom and/or 1 ring –C=C- group;

Each of up to seven R.a^5 is a substituent of a ring carbon or a ring heteroatom and:

is independently selected from H, halo, hydroxy, thiol, amine, COOH, hydrazine, cyano, saturated or unsaturated, substituted or unsubstituted $C_{1\text{-}20}$ branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, and wherein optional substituents are selected from any $C_{1\text{-}12}$ aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, such as =O, OCH_3, $CH_2Ph(OCH_3)_2$, $O(CH_2)_3CON(CH_3)$c.hex, $N(CH_2CH_2OH)_2$, c.hex, $COOCH_2CH_3$, CH_2CH_3;

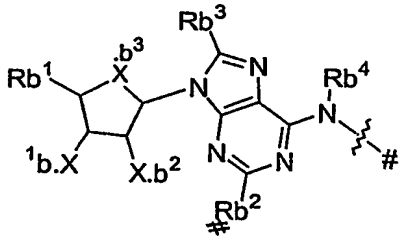
or any two or more of $R.a^5$ form a one, two or three ring fused cyclic structure, preferably comprising a fused 3 ring aryl, 5-heterocyclic, 6-heterocyclic structure having 4 ring atoms common with the fused bicyclic $Lig.a^2$structure;

and $R.a^6$ is a moiety as defined for $R.a^5$ above;

and -L.a- is as hereinbefore defined for -L- and is suitably of formula -L.I- or -L.II- as hereinbefore defined, more preferably is selected from a single bond, amino acid or amide such as a peptide or polypeptide for example gly or gly_3, alkyl of formula $-(CH_2)_n$ where n is 3 to 8, preferably 3, 4 or 6, optionally including one or more heteroatoms or unsaturated groups, such as –O- or –S- or –CH=CH- and the like:

Lig.b is of the formula Lig.b

Lig.b



wherein ring substituents $X.b^1$ and $X.b^2$ are independently selected from hydrocarbon such as alkyl or SR_X, $NR_{X.2}$ and OR_X wherein (each) R_X is selected from H, $C_{1\text{-}5}$alkyl, alkenyl;

ring heteroatom $X.b^3$ is selected from –S-, -O- and $-CH_2-$;

Rb^1 is selected from saturated or unsaturated, substituted or unsubstituted $C_{1\text{-}4}$ aliphatic, or $C_{1\text{-}3}$ alicyclic optionally including

one or more heteroatoms N, O, S, P, wherein substituent(s) are selected from one or more cycloalkyl, heterocyclic, hydroxy, oxo, halo, amine; preferably R.b^1 comprises a carbonyl substituted by H, alkyl or a linear or cyclic primary, secondary or tertiary amine, substituted C_{1-3} alkyl, cycloalkyl or amide, more preferably cyclopropyl, or $CONHC_{1-3}$alkyl such as CONHEt or CH_2OH

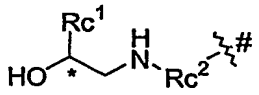
and each of R.b^2 and R.b^3 is selected from H, halo, hydroxy, thiol, amine, COOH, CHO, hydrazine, cyano or saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like, preferably from H, halo or hydroxy, preferably H or Cl;

Rb^4 is H;

-L.b- is as hereinbefore defined for –L-, more preferably saturated and unsaturated substituted or unsubstituted C_{1-12} aliphatic or C_{1-24} aromatic as defined for –L- preferably including one or more heteroatoms O, S or N, cyclic or heterocyclic groups, more preferably is of formula -L.I- or -L.II- as hereinbefore defined, most preferably is –(CH_2)m wherein m is 2 to 12, preferably 3, 4, 6 or 8, or is –(Ph-$CH_2CONH)_2$ $(CH_2)_2$-;

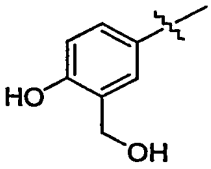
Lig.c is a non-peptide of the formula

Lig.c $HOC^*(R.c^1)CH_2NH\text{-}R.c^2\text{-}$



Where * indicates an optically active centre and

Wherein $R.c^1$ is $C_{6\text{-}14}$ aryl optionally including one or more heteroatoms selected from H, O, optionally substituted by OH, Hal eg Cl, NH_2, $NHC_{1\text{-}3}$alkyl, sulphonamide, oxoamine ($-CONH_2$) and the like, more preferably mono, di or tri substituted phenyl or quinoline wherein substituents include OH, Cl or NH_2, more preferably m-CH_2OH, p-OH phenyl, m-,p-dihydroxy phenol or m-,m-dihydroxyphenol, m-,m-diCl, p-NH_2 phenol, p-OH, m-$CONH_2$ phenol or 5-OH, 8-quinoline and the like, such as



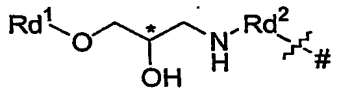
$R.c^2$ is selected from saturated or unsaturated, substituted or unsubstituted $C_{1\text{-}20}$, preferably $C_{1\text{-}12}$, branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any optionally substituted $C_{1\text{-}12}$ aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano, and the like and combinations thereof;

Preferably $R.c^2$ is selected from C_{1-6} branched or straight chain aliphatic, C_{6-10} araliphatic optionally substituted by OH and optionally including heteroatoms selected from N,O, preferably including an ether O, such as selected from $-(CH_2)_6OCH((CH_2)_3Ph)$, $CHCH_3(CH_2)_2Ph$, $CHCH_3CH_2PhOH$, $C(CH_3)_2CH_2$;

-L.c- is as hereinbefore defined for –L- and is suitably of formula -L.I- or -L.II- as hereinbefore defined, more preferably is selected from C_{1-12} alkyl, amide etc;

Lig.d is a non-peptide of the formula

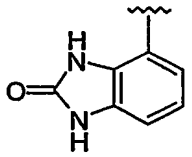
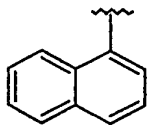
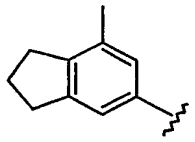
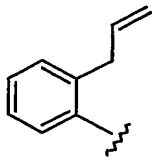
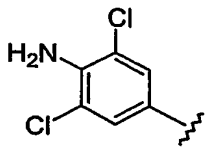
Lig.d $R.d^1\ OCH_2C^*HOHCH_2NH\text{-}R.d^2\text{-}\#$



Where * indicates an optically active centre and where # indicates the site of linking to the fluorescent tagging moiety

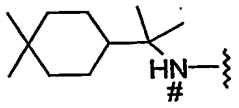
Wherein $R.d^1$ is saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo, cyano;

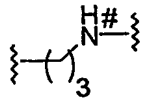
Preferably R.d[1] is substituted or unsubstituted C_{1-24} aralkyl or heteroaralkyl, including single ring and fused ring systems with (hetero)aryl or cycloalkyl rings, wherein optional substituents include C_{1-6} alkyl, alkoxy, ether, carbonyl, alkenyl, amine, amide each optionally carbonyl, amide, halo or OH substituted, or halo such as chloro or OH, preferably R.d[1] is unsubstituted or substituted alkyl, alkenyl, halo, amine, amide, carbonyl, ketone, ether substituted phenyl or naphthyl, illustrated as follows, most preferably mono-, di-, tri- or tetra substituted mono or polycyclic fused aryl or cycloaryl or heterocycloaryl such as phenyl, carbazole or structures shown below or spiro ring systems, most preferably mono-, di-, tri- or tetra alkoxyalkyl, alkoxyalkoxyalkyl or CF_3 substituted phenyl or unsubstituted or monosubstituted naphthalene or 5,6 ring systems most preferably of the structures:

R.d[2] is substituted or unsubstituted amine, saturated or unsaturated, substituted or unsubstituted C_{1-12} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P; wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine,

hydrazine, oxo, cyano, and the like, more preferably amine, C_{1-6} branched or straight chain alkyl optionally including ether O, and optionally substituted by C_{6-10} aryl, for example of the formula:





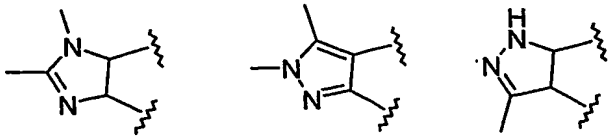
i.pr, i.bu, CH_2CH_2O (m-$CONH_2$, p-OH) phenol, CH_2CH_2O (o-OCH_3 phenol

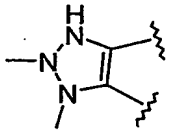
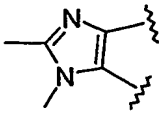
-L.d- is as hereinbefore defined for –L- and is suitably of formula -L.I- or -L.II- as hereinbefore defined, more preferably is a single bond or is as hereinbefore defined for -L.a-;

Lig.e comprises a cell permeant moiety or is associated with a cell permeant L or Fl moiety and is suitably of the formula , in either of the following forms given:

Lig.e[1]

wherein h is selected from



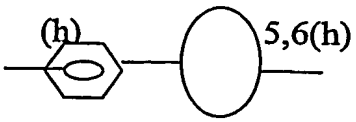
each optionally substituted by R.e^3 – R.e^4,wherein R.e^1 – R.e^4 are as R.a^1 – R.a^4 defined above or in which R.e^3 is C_{5-9}linear or branched alkyl, optionally mono or multi hydroxy or halo substituted or is aryl optionally substituted by alkoxy, sulfonyl and the like eg ortho-OEt, meta-SO_2N  NCH_3

each X is independently selected from H, =O, -OR.e^2, =N, HN, NR.e^5, HR.e^6 , and aryl optionally substituted by ether; or X is aryl optionally alkyl or alkoxy substituted such as Ph-ortho-$OCH_2CH_2CH_3$;

and where R.e^5 is as defined above for R.e^1 above or together with R.e^1 forms a fused cyclic ring together with the adjacent ring N atom; preferably 1 or 2 fused 5 membered cyclic rings;

and R.e^6 is as defined above for R.e^1 above or is selected from optionally substituted phenyl wherein optional substituents include ether such as o-ethoxy or o-propoxy, alkyl, OH and the like, sulphonyl, carbonyl and the like substituted by heterocyclic, or cyclic C_{5-8} alkyl such as methyl, piperazinyl, sulphonyl and the like;

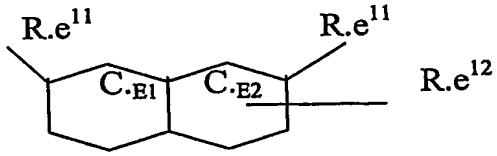
or Lig.e is of the formula Lig.e^2

Lig.e^2 

Wherein each spiro ring optionally comprises zero or one or more heteroatoms h which are preferably N, more preferably  (h) comprises zero or 1 N heteroatom and  5,6(h) comprises zero, 1 or 2 N heteroatoms and is unsaturated or comprises one or two –C=C- or -C=N- groups;

and wherein each ring is optionally substituted by one or more oxo, CO, COOH, C_{1-6} alkyl or linear or cyclic alkoxy such as methoxy, ethoxy or cyclopentyloxy optionally substituted by one or more oxo, CO, COOH, CN, or C_{1-6} alicyclic or amine groups, amine or one or more spiro or fused heterocycles;

or Lig.e is of the formula $Lig.e^3$

$Lig.e^3$ 

Wherein each of $C._{E1}$ and $C._{E2}$ is independently selected from aryl, heteroaryl, cyloalkyl and heterocyclic, more preferably from phenyl, or aryl containing 1 or 2 ring heteroatoms, or heterocyclic containing 1 ring heteroatom and/or 1 ring –C=C- group;

Each of up to seven $R.e^{11}$ is a substituent of a ring carbon or a ring heteroatom and:

is independently selected from saturated or unsaturated, substituted or unsubstituted C_{1-20} branched or straight chain aliphatic, aromatic, alicyclic and combinations thereof, any of which may comprise one or more heteroatoms selected from N, O, S, P, and wherein optional substituents are selected from any C_{1-12} aliphatic, aromatic or alicyclic substituents any of which may comprise one or more heteroatoms as hereinbefore defined, hydroxy, thiol, halo, amine, hydrazine, oxo,

cyano, and the like, such as =O, OCH_3, $CH_2Ph(OCH_3)_2$, $O(CH_2)_3CON(CH_3)$c.hex, $N(CH_2CH_2OH)_2$, c.hex, $COOCH_2CH_3$, CH_2CH_3;

or any two or more of R.e[11] form a one, two or three ring fused cyclic structure, preferably comprising a fused 3 ring aryl, 5-heterocyclic, 6-heterocyclic structure having 4 ring atoms common with the fused bicyclic Lig.e[3] structure;

and R.e[12] is a moiety as defined for R.e[11] above.

12. Kit as claimed in any of Claims 1 to 12 wherein -Fl is selected from dyes in particular including fluorescein, fluorescein derivatives including FITC, and fluorescein-like molecules such as Oregon Green™ and its derivatives, Texas red™, 7-nitrobenz-2-oxa-1,3-diazole (NBD) and derivatives thereof, coumarin and derivatives, naphthalene including derivatives of dansyl chloride or its analogues or derivatives, Cascade Blue™, EvoBlue and fluorescent derivatives thereof, pyrenes and pyridyloxazole derivatives, the cyanine dyes, the dyomics (DY dyes and ATTO dyes) and fluorescent derivatives thereof, the Alexafluor dyes and derivatives, BDI dyes including the commercially available Bodipy™ dyes, erythosin, eosin, pyrenes, anthracenes, acridines, fluorescent phycobiliproteins and their conjugates and fluoresceinated microbeads, Rhodamine and fluorescent derivatives thereof including Rhodamine Green™ including the tetramethylrhodamines, X-rhodamines and Texas Red derivatives, and Rhodol Green™, coupled to amine groups using the isocyanate, succinimidyl ester or dichlorotriazinyl-reactive groups and other red, blue or green absorbing fluorescent dyes in particular red absorbing dyes as reviewed in Buschmann V et al, Bioconjugate Chemistry (2002), ASAP article.

13. Kit as claimed in any of Claims 1 to 12 wherein -Fl comprises fluorescein, Texas Red ™, Cy5.5 or Cy5 or analogues thereof, BODIPY ™

630/650 and analogues thereof, DY- 630, DY-640, DY-650 or DY-655 or analogues thereof, ATTO 655 or ATTO 680 or analogues thereof, EvoBlue 30 or analogues thereof, Alexa 647 or analogues thereof.

14. Kit as claimed in any of Claims 12 to 14 wherein –Fl comprises any of the therein cited commercially available fluorophores modified to form a derivative or group of derivatives suitable for visualising ligand binding in a kit as hereinbefore defined preferably wherein Fl- is Fl'-t- wherein –t- comprises functionality derived linking to a precursor ligand as hereinbefore defined and may optionally comprise a proximal unsaturated or aryl moiety, comprising a medial short, medium or long chain alkynyl or cycloalkyl moiety and comprising a moiety derived from linking via a reactive group as hereinbefore defined such as carboxyl, sulphonate or as a heteroatom such as O or S or methylene derived from linking at an alkylhalide such as methylbromide, haloacetamide, sulphonate ester or the like electrophilic group.

15. Kit as claimed in any of Claims 12 to 14 wherein -Fl comprising a BODIPY ™ structure is characterised by a dipyrromethene boron difluoride core, optionally modified by one or two fused rings, optionally substituted by one or several substituents such as alkyl, alkoxy, aryl, heterocyclic and the like, wherein one substituent –t- is adapted for linking as hereinbefore defined to a ligand precursor as hereinbefore defined, the substituent –t- optionally comprising a proximal unsaturated or aryl moiety, comprising a medial short, medium or long chain alkynyl or cycloalkyl moiety and comprising a moiety derived from linking via a reactive group as hereinbefore defined such as carboxyl, sulphonate or as a heteroatom such as O or S or methylene derived from linking at an alkylhalide such as methylbromide, haloacetamide, sulphonate ester or the like electrophilic group

16. Process for the preparation of a kit as hereinbefore defined in any of Claims 1 to 15 comprising the reaction of one or each of a plurality of ligand precursors of formula

$Lig\text{-}L\text{-}Z_L$

comprising a reactive site wherein $-Z_L$ is a leaving group

with a plurality of analytical tagging substrates of formula

$Tag\text{-}Z_T$

comprising a reactive site wherein $-Z_T$ is a leaving group;

wherein the or each $Lig\text{-}L\text{-}Z_L$ is capable of reaction with a plurality of $Tag\text{-}Z_T$, to form a plurality of compounds Lig-L-Tag.

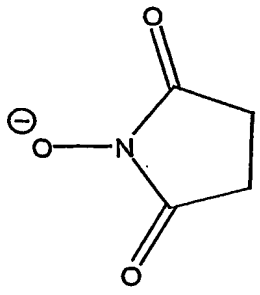
17. Process as claimed in Claim 16 wherein a some or all of $Tag\text{-}Z_T$ is a compound $Fl\text{-}Z_F$ comprising a reactive site wherein Z_F is a leaving group as hereinbefore defined for Z_T and comprises any known or novel fluorophore, whereby the process is a process for preparing a kit comprising a plurality of compounds of which one or more or all of which are of formula Lig-L-Fl.

18. Process as claimed in any of Claims 16 and 17 wherein ligand $Lig\text{-}L\text{-}Z_L$ and fluorophore $Fl\text{-}Z_F$ have suitable reactive end group functionalities for linking, as hereinbefore defined, for example selected from combinations of electrophilic ($Lig\text{-}L\text{-}Z_L$ or $Fl\text{-}Z_F$ usually $Fl\text{-}Z_F$) and nucleophilic ($Lig\text{-}L\text{-}Z_L$ or $Fl\text{-}Z_F$ usually $Lig\text{-}L\text{-}Z_L$) groups such as:

$Fl\text{-}Z_F$	$Lig\text{-}L\text{-}Z_L$	Lig-L-Fl	$-Z_F$	$-Z_L$
Electrophilic	Nucleophilic	Resulting covalent linkage	leaving gp	leaving gp
Carboxylic acid	alcohol	ester	-OH	-H
Carboxylic acid	amine	carboxamide	-OH	-H
Carboxylic acid	hydrazine	hydrazide	-OH	-H

Alkyl halide	alcohol	ether	-Hal	-H
Alkyl halide	thiol	thioether	-Hal	-H
Alkyl halide	amine	alkylamine	-Hal	-H
Alkyl halide	COOH	ester	-Hal	-H
Haloacetamides	thiols	thioethers	-Hal	-H
Sulphonate esters	amines	alkyl amines	RSO_3-	-H
Sulphonate esters	alcohols	ethers	RSO_3-	-H
Sulphonate esters	thiols	thioethers	RSO_3-	-H
Sulphonyl halides	amines	sulphonamides	-Hal	-H
Sulphonyl halides	alcohols	sulphonate esters	-Hal	-H
Succinimide ester	alcohols	esters	-OSu*	-H
Succinimide ester	alkoxides	esters	-OSu*	-H or M^+
Succinimide ester	thiols	thioesters	-OSu*	-H
Succinimide ester	amine	carboxamide	-OSu*	-H
Succinimide ester	hydrazine	hydrazide	-OSu*	-H

Wherein * is



19. Process as claimed in Claims 16 to 18 wherein Lig-L-Z_L comprises no protecting group and is capable of reaction with Tag-Z_T without degradation of functionality by choice of reaction and of respective reactive sites; or Lig-L-Z_L comprises one or more protecting groups which are adapted for removal

under ambient conditions, for example under neutral pH, room temperature or the like.

20. Fluorescently tagged ligand Lig-L-Fl wherein Lig-, -L- and –Fl are as hereinbefore defined in any of Claims 1 to 15 wherein the compound is associated with information relating to its pharmacological properties in the form of Spectral Properties given as Excitation Max and Emission Max, Fluorescence Lifetime and Emission quantum yield and Pharmacology defined in terms of cells expressing a biogenic amine GPCR receptor as hereinbefore defined or expressing an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases, and given as the Inhibition or Antagonism of receptor binding or of receptor functionality together with a value for the Inhibition (pK_B) or Antagonism (pK_I), and optionally together with images of the pharmacological binding illustrating the defined inhibition or antagonism.

21. Fluorescently tagged ligand Lig-L-Fl as claimed in Claim 20 associated with information relating to its pharmacological properties wherein pharmacology is defined in terms of a cell or protein wherein the cell comprises a GPCR or the protein is a G-protein, preferably in terms of a CHO cell comprising GPCR receptors selected from adenosine A_1-, A_{2A}-, A_{2B}- and A_3-receptors, β_1, β_2- and β_3- adrenoceptors, or comprises an inhibitor of an intracellular enzyme such as cyclic nucleotide phosphodiesterases, more preferably in terms of CHO-cells expressing human adenosine A_1-receptor or beta-adrenoceptor or an inhibitor of an intracellular enzyme such as an inhibitor of intracellular enzymes PDE 1-5 and which is given as the Inhibition of ^{3}H-DPCPX binding (membranes or whole cells) (pK_B) and /or Antagonism of NECA-stimulated cAMP accumulation (pK_I), Antagonism of NECA-stimulated inositol phosphate accumulation (pK_I), and/or the

Stimulation of cellular responses such as subcellular responses (EC_{50}) and/or the Inhibition of cyclic nucleotide phosphodiesterase activity (pK_B).

22. Fluorescently tagged ligand of the formula

Lig – L - Fl

Wherein Lig- comprises a biogenic amine GPCR receptor ligand selected from a) an adenosine receptor antagonist b) an adenosine receptor agonist c) a beta-adrenoceptor agonist and d) a beta-adrenoceptor antagonist; or Lig- comprises e) an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases; or a derivative or analogue thereof;

-L- is a single bond or is any linking moiety which may be monomeric, oligomeric having oligomeric repeat of 2 to 30 or polymeric having polymeric repeat in excess of 30 up to 300;

and Fl is any known or novel fluorophore, preferably as hereinbefore defined;

with the proviso that:

a) when Lig is XAC and L is a single bond (ie n=0) or L is gly and n=3, Fl is not fluorescein; or

when Lig is XAC and L is a single bond and n=1, Fl is not fluorescein or NBD;

b) when Lig is ADAC and L is a single bond, Fl is not fluorescein, NBD or Rhodamine; or

when Lig is NECA (incorporating the moiety $-(CH_2)m$) and L is a single bond, when m is 2,4,6,8 or 10 then Fl is not NBD, or when m is 3,4,6,8,10 or 12 then Fl is not dansyl; or

c) -

d) when Lig is CGP12177 and L is mono amine menthane, Fl is not BODIPY® TMR; or

when Lig is CGP12177 and L is 1,1,4,4-tetramethyl butylamine, Fl is not BODIPY® FL, FITC, eosin or erythosin; or

when Lig is CGP12177 and L is a single bond, Fl is not NBD; or

when Lig is alprenolol and L is $-C(CH_3)_2-$ or a single bond, Fl is not NBD

and wherein additionally

a) when Lig is XAC and L is a single bond Fl is not BODIPY ™ 630/650; or

b) when Lig is ABEA and L is a single bond Fl is not BODIPY™ 630/650.

23. Ligand of formula Lig-L-Fl as claimed in Claim 22 selected from formulae Lig.aL.a-Fl.a – Lig.eL.eFl.e wherein –L- are as hereinbefore defined in any of Claims 1 to 15:

Lig.a-L.a – Fl.a

wherein Lig.a comprises xanthine like structures including XAC, theophyline, caffeine, theobromine, dyphilline, enprofylline and the like; or fused biaryl structures including papaverine, cilostamide and the like; and analogues thereof;

Fl is BODIPY 630/650 and the moieties are directly or indirectly covalently linked via the XAC-8-substituent which preferably terminates with a diamino moiety;

Lig.b- L.b – Fl.b

wherein Lig.b comprises adenosine like structures including ADAC, ABEA, NECA and analogues thereof;

and Fl = BODIPY 630/650 and L.b is preferably a diamino or biphenylamide as hereinbefore defined;

Lig.c- L.c – Fl.c

wherein Lig.c comprises ethanolamine structures including salmeterol, salbutamol, terbutaline, quinprenaline, bambuterol, fenoterol, reprotolol, tulobuterol, clenbuterol and analogues thereof; L.c is as hereinbefore defined

and Fl is selected from BODIPY 630/650);

Lig.d- L.d– Fl.d

wherein Lig.d comprises oxypropanolamine structures including CGP12177, propranolol, practolol, acebutalol, betaxolol, ICI 118551, alprenolol, celiprolol (celectol), metoprolol (betaloc), CGP20712A, atenolol, bisoprolol, misaprolol, carvedilol, bucindolol, esmolol, labetalol, nadolol, nebivolol, oxprenolol, xamoterol, pindolol, sotalol, timolol and analogues thereof; and

L.d is cyclic (di)amine or linear (di)amine as hereinbefore defined and

Fl = BODIPY 630/650;

Lig.e- L.e– Fl.e

wherein Lig.e comprises xanthine like structures including XAC, theophylline, caffeine, theobromine, dyphilline, enprofylline, sildenafil, EHNA (erythro-9-(2-hydroxyl-3-nonyl)adenine), zaprinast and the like; or spiro bicyclic structures including bypyridines such as amrinone, imidazolines such as CI930, dihydropyridazinones such as indolan, rolipram, SB207499, and the like; or fused biaryl structures including papaverine, dihydroquinilones such as cilostamide, dipyridamole, vinpocetine and the like and analogues thereof; L.e is as hereinbefore defined;

and Fl = BODIPY 630/650.

24. Ligand as claimed in Claim 22 or 23 wherein the mioety Lig-L- is selected from formulae Lig.a-L.a- – Lig.e-L.e- as hereinbefore defined in Claim 11

with the proviso that

in Lig.a^1 -L.a^1- when each of R.a^1 and R.a^2 is propyl, R.a^3 is H and R.a^4 is –Ph-$OCH_2CONH(CH_2)_2NH$-, and L is a single bond or gly_3 or NCS then Fl is not fluorescein; or

when each of R.a^1 and R.a^2 is propyl, R.a^3 is H and R.a^4 is –Ph-$OCH_2CONH(CH_2)_2NH$-, and L.a is NCS then Fl is not NBD; or

or with the proviso that in Lig.b-L.b-

When R.b^1 is CH_2OH, R.b^2 and R.b^3 are H and L is –$(Ph\text{-}CH_2CONH)_2(CH_2)_2$- then Fl is not fluorescein, NBD or Rhodamine; or

When R.b^1 is CONHEt, R.b^2 and R.b^3 are H and L is –(CH_2)m where m is 2,4,6,8,10 then Fl is not NBD or where m is 3,4,6,8,10,12 then Fl is not dansyl;

or with the proviso that in Lig.d-L.d when Lig.d is CGP12177 and R.d^2 is monoamine menthane, then Fl is not BODIPY ® TMR or when R.d^2 is $–C(CH_3)_2(CH_2)_2C(CH_3)_2NH$- then Fl is not BODIPY ® FL or when R.d^2 is $–C(CH_3)_2(CH_2)_2C(CH_3)_2NHCSNH$- then Fl is not FITC, eosin or erythosin, or

when R.d^1 is o-prop-2-enyl phenyl and R.d^2 is $C(CH_3)_2$ then Fl is not NBD;

and additionally in a) when each of R.a^1 and R.a^2 is propyl, R.a^3 is H and R.a^4 is –Ph-$OCH_2CONH(CH_2)_2NH$-, and L.a is a single bond then Fl is not BODIPY ® 630/650; or in

b) where m is 4 then Fl is not BODIPY ® 630/650.

25. Process for the preparation of tagged ligands of formula Lig-L-Tag, suitably fluorescently tagged ligands of formula Lig-L-Fl as hereinbefore defined in any of Claims 1 to 15 or 20 to 24 comprising: reacting reactive ligand Lig-L-Z_L with reactive tag Tag-Z_T, suitably reactive fluorophore Fl-Z_F comprising a reactive group having leaving group Z_F or Z_T wherein reactive

groups are selected so as to enable reaction with a fully deprotected ligand ie without the need for protecting groups or so as to enable reaction with protecting groups present which may be removed under mild conditions.

26. Process as claimed in Claim 25 wherein reactive groups Z_L and Z_F or Z_T are selected so as to enable reaction with a fully deprotected ligand, for example Z_L comprises amine or alcohol or thiol and Z_T or Z_F comprises succinimide ester; or alternatively a protecting group which may be removed under mild conditions comprises benzyloxycarbonyl and the like which are removed at ambient conditions such as room temperature or under conditions which do not prejudice functional groups such as the glycosidic group in Lig.b.

27. Process as claimed in any of Claims 25 and 26 comprising a), b), c), d), e) reacting the unprotected primary alkyl amine group of Lig.a-L.a-Z.a, Lig.b-L.b-Z.b, Lig.c-L.c-Z.c, Lig.d-L.d-Z.d or Lig.e-L.e-Z.e as hereinbefore defined with Fl-Z_F comprising a reactive succinimidyl ester group in solvent at ambient temperature without need for subsequent deprotection.

28. Process for the preparation of a reactive ligand Lig-L-Z_L as hereinbefore defined in any of Claims 26 and 27 comprising: obtaining where commercially available or preparing the ligand Lig-, by routes as known in the art, and introducing linker -L-Z_L and removing any protecting group present during the synthesis, optionally replacing with a protecting group which may be removed under ambient conditions.

29. Process as claimed in Claim 28 comprising a), e) ring closure of 5,6-diamino-1,3-dialkyl uracil with the appropriate substituted aldehyde under acid conditions with ferric chloride, or obtaining compounds by the process of Jacobsen *et al* as hereinbefore referred; or as referred in publications described

by Galatin (Viagra)
http://views.vcu.edu/~pgalatin/viagra.html;

b) reacting Lig.b- comprising a protected inosine derivative with chlorinating agent and linking the chloro derivative with the amine group of a suitably protected amine reactive linker H–L-P_L wherein P_L comprises *N*-benzyloxycarbonyl- to form Lig-L-P_L and removing P_L to generate Lig-L-Z_L; preferably R.b^1 comprises a OH terminating group and protected inosine comprises Acyl protecting groups or R.b^1 comprises a stable group such as amine or amide and protected inosine comprises 2,2-dimethoxypropane protecting group; preferably the protected inosine is reacted with oxidising agent and protected alkylamine which is an *N*-alkylcarboxamide with removal of amine protecting group to generate a reactive ligand;

c), d) reacting m-hydroxybenzaldehyde with dioxane to form a protected diol, converting aldehyde to epoxy, reacting a suitably protected linker such as Boc-L.c-H which opens up the epoxy and supplies Lig-L-P_L and deprotecting under acid conditions.

30. Novel ligand precursor Lig-L-Z_L useful for linking to any suitable tag Tag-Z_T, in particular any suitable fluorophore Fl-Z_F, wherein Lig-, -L- and –Z_L and Tag, Fl, Z_T and Z_F are as hereinbefore defined in any of Claims 1 to 15, with the proviso that when Lig is Lig.a as hereinbefore defined and

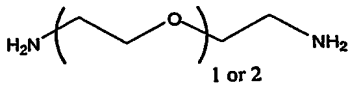
Z_L is -CO_2H, R.a^1 and R.a^2 are CH_3 or n-C_3H_7, then R.a^4 is not H, CH_2CO_2H, or $CH_2CONHBzCH_3$; or when

Z_L is OH and R.a^1 and R.a^2 are n-C_3H_7, then R.a^4 is not $CH_2CONHBz$; or when

Z_L is NH_2 and R.a^1 and R.a^2 are n-C_3H_7, then R.a^4 is not CH_2CO-, CH_2CONH or compounds as disclosed in Jacobsen *et al* as hereinbefore referred.

31. Linker for linking any known biogenic amine G protein coupled receptor ligand and fluorophore comprising an ether preferably a PEG ether as

hereinbefore defined, and retaining pharmacological activity, more preferably of formula

Diamino-PEG ether 

or $H_2N(CH_2CH_2O)pCH_2CH_2NH_2$

wherein p is a whole number integer selected from 1 or 2 to 30, eg 1 or 2 to 20, or represents a polymeric repeat of the order of 30 up to 300.

32. Novel fluorophore of formula Fl-Z_F or Fl-t-Z_F useful for linking to any suitable ligand of formula Lig-L-Z_L,

wherein Fl, Z_F, -t-, Lig, L and Z_L are as hereinbefore defined in any of Claims 1 to 15, preferably wherein –t- comprises -(PEG ether)n where n= 1 or 2 to 30, or 30 to 300 as hereinbefore defined.

33. Method for cell binding and visualisation comprising contacting a fluorescent ligand as hereinbefore defined in any of Claims 1 to 15 or 20 to 24 with a sample in manner to facilitate binding thereof, and detecting changes in fluorescence.

34. Method as claimed in Claim 3 wherein a sample comprises cell material, selected from cells, cell extracts, cell homogenates, purified or reconstituted proteins, recombinant proteins or synthesised proteins and the like. Samples comprising cell material may be derived from plants, animals, fungi, protists, bacteria, archae or cell lines derived from such organisms. Animal or plant cells used to prepare the sample may be healthy or disfunctional and are optionally used in the diagnosis of a disease such as leukaemia or cancer. In a preferred embodiment of the invention the sample comprises mammalian cells, extracts and homogenates thereof.

35. Method as claimed in any of Claims 33 and 34 wherein the cell or protein is a cell comprising a GPCR or the protein is a G-protein, preferably the sample is a CHO cell comprises GPCR receptors selected from adenosine A_1-, A_{2A}-, A_{2B}- and A_3-receptors, β_1, β_2- and β_3- adrenoceptors, or comprises inhibitors of intracellular enzymes such as cyclic nucleotide phosphodiesterases, more preferably CHO-cells expressing human adenosine A_1-receptor or beta-adrenoceptor or an inhibitor of an intracellular enzyme such as an inhibitor of intracellular enzymes PDE 1-5.

36. Kit, fluorescently tagged ligand, ligand precursor, fluorophore precursor, processes for the preparation thereof, and method for cell binding with use thereof substantially as hereinbefore described in the Examples and or illustrated in the Figures.

ABSTRACT

Kit comprising a plurality of tagged non-peptide ligands of formula

Lig-L-Tag

in each of which a precursor ligand is linked to one of a plurality of different tags or each of a plurality of precursor ligands are linked to a plurality of different tags

Wherein Lig- comprises a biogenic amine GPCR receptor ligand selected from a) an adenosine receptor antagonist b) an adenosine receptor agonist c) a beta-adrenoceptor agonist and d) a beta-adrenoceptor antagonist; or Lig- comprises e) an inhibitor of an intracellular enzyme such as an inhibitor of cyclic nucleotide phosphodiesterases; or a derivative or analogue thereof;

-L- is a single bond or is any linking moiety which may be monomeric, oligomeric having oligomeric repeat of 2 to 30 or polymeric having polymeric repeat in excess of 30 up to 300;

and wherein each -Tag is any known or novel tagging substrate; preferably wherein one or more or each -Tag is an entity -Fl and comprises any known or novel fluorophore, whereby the kit comprises a plurality of compounds of which one or more or all of which are of formula Lig-L-Fl; known and novel fluorescently tagged ligands; novel ligand precursors; novel methods for the preparation thereof; and the use thereof in cell binding studies.

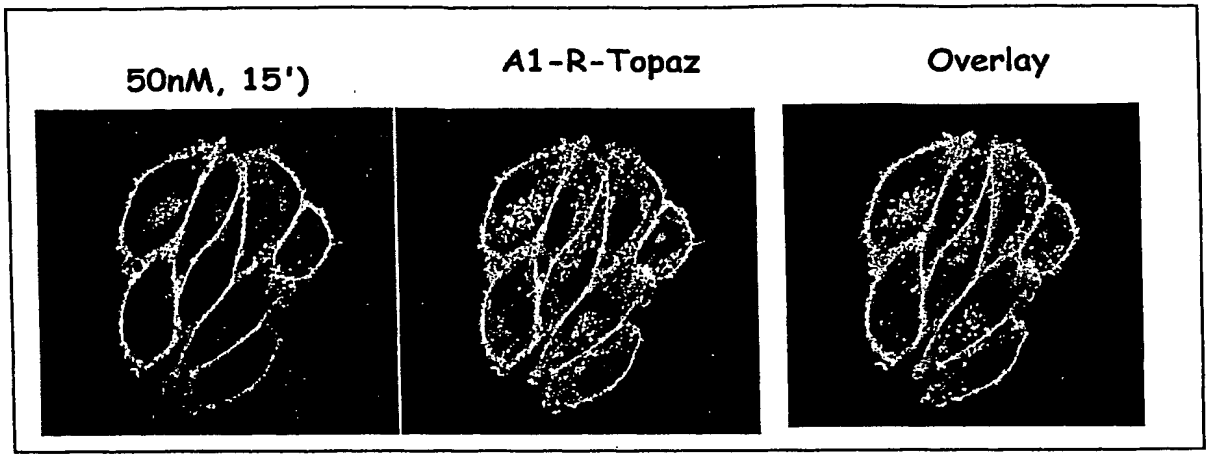


Fig 1. Binding of BODIPY-XAC to CHO-K1 cells expressing the human A1-receptor with a Green fluorescent protein tag attached to the C terminus. (a) binding of red ligand; (b) location of A1-GFP receptor and (c) co-localisation (yellow) of two signals obtained via confocal microscopy.

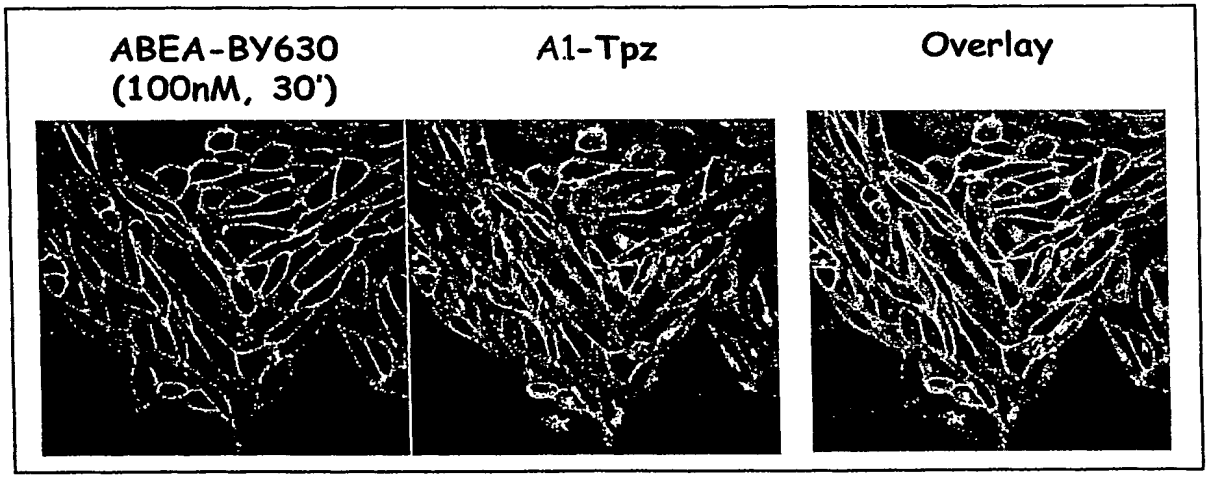
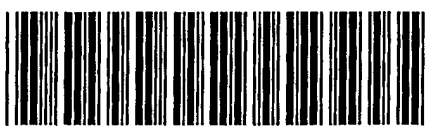


Fig 2. Binding of BODIPY-ABEA to CHO-K1 cells expressing the human A1-receptor with a Green fluorescent protein tag attached to the C terminus. (a) binding of red ligand; (b) location of A1-GFP receptor and (c) co-localisation (yellow) of two signals obtained via confocal microscopy



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